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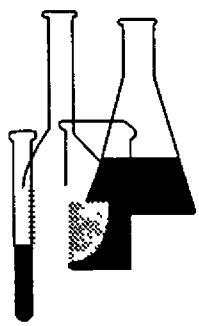
Health Effects Test Guidelines OPPTS 870.5100 Bacterial Reverse Mutation Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U.S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.5100 Bacterial reverse mutation test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5100 Escherichia coli WP2 and WP2 uvrA reverse mutation assays, OPPTS 40 CFR 798 5265 The salmonella typhimurium reverse mutation assay and OECD 471 and 472, Bacterial Reverse Mutation Test

(b) **Purpose.** (1) The bacterial reverse mutation test uses amino-acid requiring strains of *Salmonella typhimurium* (*S typhimurium*) and *Escherichia coli* (*E coli*) to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (see references in paragraphs (g)(1), (g)(2), and (g)(3) of this guideline) The principle of this bacterial reverse mutation test is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain

(2) Point mutations are the cause of many human genetic diseases and there is substantial evidence that point mutations in oncogenes and tumour suppressor genes of somatic cells are involved in tumour formation in humans and experimental animals The bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of structures is available for bacterial reverse mutation tests and well-established methodologies have been developed for testing chemicals with different physico-chemical properties, including volatile compounds.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline.

Reverse mutation test in either *Salmonella typhimurium* or *Escherichia coli* detects mutation in an amino-acid requiring strain (histidine or tryptophan, respectively) to produce a strain independent of an outside supply of amino-acid

Base pair substitution mutagens are agents that cause a base change in DNA In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome

Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA

Initial considerations. (1) The bacterial reverse mutation test utilizes prokaryotic cells, which differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals

(2) The bacterial reverse mutation test is commonly employed as an creen for genotoxic activity and, in particular, for point mutation-inducing activity An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests There are examples of mutagenic agents which are not detected by this test, reasons for these shortcomings can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability On the other hand, factors which enhance the sensitivity of the bacterial reverse mutation test can lead to an overestimation of mutagenic activity

(3) The bacterial reverse mutation test may not be appropriate for the evaluation of certain classes of chemicals, for example highly bactericidal compounds (e.g , certain antibiotics) and those which are thought (or known) to interfere specifically with the mammalian cell replication system (e g., some topoisomerase inhibitors and some nucleoside analogues). In such cases, mammalian mutation tests may be more appropriate

(4) Although many compounds that are positive in this test are mammalian carcinogens, the correlation is not absolute It is dependent on chemical class and there are carcinogens that are not detected by this test because they act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells

(e) **Test method—(1) Principle.** (i) Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system In the plate incorporation method, these suspensions are mixed with an overlay agar and plated immediately onto minimal medium In the preincubation method, the treatment mixture is incubated and then mixed with an overlay agar before plating onto minimal medium For both techniques, after 2 or 3 days of incubation, revertant

colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates

(ii) Several procedures for performing the bacterial reverse mutation test have been described Among those commonly used are the plate incorporation method (see references in paragraphs (g)(1), (g)(2), (g)(3), and (g)(4) of this guideline), the preincubation method (see references in paragraphs (g)(2), (g)(3), (g)(5), (g)(6), (g)(7), and (g)(8) of this guideline), the fluctuation method (see references in paragraphs (g)(9) and (g)(10) of this guideline), and the suspension method (see reference in paragraph (g)(11) of this guideline) Suggestions for modifications for the testing of gases or vapours have been described (see reference in paragraph (g)(12) of this guideline)

(iii) The procedures described in this guideline pertain primarily to the plate incorporation and preincubation methods Either of them is acceptable for conducting experiments both with and without metabolic activation Some compounds may be detected more efficiently using the preincubation method These compounds belong to chemical classes that include short chain aliphatic nitrosamines, divalent metals, aldehydes, azo-dyes and diazo compounds, pyrollizidine alkaloids, allyl compounds and nitro compounds (see reference in paragraph (g)(3) of this guideline). It is also recognized that certain classes of mutagens are not always detected using standard procedures such as the plate incorporation method or preincubation method These should be regarded as "special cases" and it is strongly recommended that alternative procedures should be used for their detection The following "special cases" could be identified (together with examples of procedures that could be used for their detection) azo-dyes and diazo compounds (see references in paragraphs (g)(3), (g)(5), (g)(6), and (g)(13) of this guideline), gases and volatile chemicals (see references in paragraphs (g)(12), (g)(14), (g)(15), and (g)(16) of this guideline), and glycosides (see references in paragraphs (g)(17) and (g)(18) of this guideline) A deviation from the standard procedure needs to be scientifically justified

(2) **Description**—(i) **Preparations**—(A) **Bacteria.** (*1*) Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^9 cells per ml). Cultures in late stationary phase should not be used The cultures used in the experiment should contain a high titre of viable bacteria. The titre may be demonstrated either from historical control data on growth curves, or in each assay through the determination of viable cell numbers by a plating experiment.

(*2*) The culture temperature should be 37 °C

(*3*) At least five strains of bacteria should be used These should include four strains of *S typhimurium* (TA1535, TA1537 or TA97a or TA97; TA98, and TA100) that have been shown to be reliable and

reproducibly responsive between laboratories These four *S typhimurium* strains have GC base pairs at the primary reversion site and it is known that they may not detect certain oxidising mutagens, cross-linking agents and hydrazines Such substances may be detected by *E coli* WP2 strains or *S typhimurium* TA102 (see reference in paragraph (g)(19) of this guideline) which have an AT base pair at the primary reversion site Therefore the recommended combination of strains is

(*i*) *S typhimurium* TA1535

(*ii*) *S typhimurium* TA1537 or TA97 or TA97a

(*iii*) *S typhimurium* TA98

(*iv*) *S typhimurium* TA100

(*v*) *E coli* WP2 *uvrA*, or *E coli* WP2 *uvrA* (pKM101), or *S typhimurium* TA102

In order to detect cross-linking mutagens it may be preferable to include TA102 or to add a DNA repair-proficient strain of *E coli* [e g , *E.coli* WP2 or *E coli* WP2 (pKM101)]

(*4*) Established procedures for stock culture preparation, marker verification and storage should be used The amino-acid requirement for growth should be demonstrated for each frozen stock culture preparation (histidine for *S typhimurium* strains, and tryptophan for *E coli* strains) Other phenotypic characteristics should be similarly checked, namely the presence or absence of R-factor plasmids where appropriate [i e ampicillin resistance in strains TA98, TA100 and TA97a or TA97, WP2 *uvrA* and WP2 *uvrA* (pKM101), and ampicillin + tetracycline resistance in strain TA102], the presence of characteristic mutations (i e rfa mutation in *S. typhimurium* through sensitivity to crystal violet, and *uvrA* mutation in *E coli* or *uvrB* mutation in *S typhimurium*, through sensitivity to ultraviolet light) (see references in paragraphs (g)(2) and (g)(3) of this guideline) The strains should also yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.

(B) **Medium.** An appropriate minimal agar (e g., containing Vogel-Bonner minimal medium E and glucose) and an overlay agar containing histidine and biotin or tryptophan, to allow for a few cell divisions, should be used (see references in paragraphs (g)(1), (g)(2), and (g)(9) of this guideline)

(C) **Metabolic activation.** Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (see references in para-

graphs (g)(1) and (g)(2) of this guideline) or a combination of phenobarbitone and β-naphthoflavone (see references in paragraphs (g)(18), (g)(20), and (g)(21) of this guideline) The post-mitochondrial fraction is usually used at concentrations in the range from 5 to 30 percent v/v in the S9-mix The choice and condition of a metabolic activation system may depend upon the class of chemical being tested In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction For azo-dyes and diazo-compounds, using a reductive metabolic activation system may be more appropriate (see references in paragraphs (g)(6) and (g)(13) of this guideline)

(D) **Test substance/preparation.** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the bacteria Liquid test substances may be added directly to the test systems and/or diluted prior to treatment Fresh preparations should be employed unless stability data demonstrate the acceptability of storage

(ii) **Test conditions**—(A) **Solvent/vehicle.** The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the bacteria and the S9 activity (see reference in paragraph (g)(22) of this guideline) If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first When testing water-unstable substances, the organic solvents used should be free of water

(B) **Exposure concentrations.** (*1*) Amongst the criteria to be taken into consideration when determining the highest amount of test substance to be used are cytotoxicity and solubility in the final treatment mixture. It may be useful to determine toxicity and insolubility in a preliminary experiment. Cytotoxicity may be detected by a reduction in the number of revertant colonies, a clearing or diminution of the background lawn, or the degree of survival of treated cultures The cytotoxicity of a substance may be altered in the presence of metabolic activation systems. Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eye. The recommended maximum test concentration for soluble non-cytotoxic substances is 5 mg/plate or 5µl/plate For non-cytotoxic substances that are not soluble at 5mg/plate or 5µl/plate, one or more concentrations tested should be insoluble in the final treatment mixture Test substances that are cytotoxic already below 5mg/plate or 5µl/plate should be tested up to a cytotoxic concentration The precipitate should not interfere with the scoring

(*2*) At least five different analysable concentrations of the test substance should be used with approximately half log (i e $\sqrt{10}$) intervals be-

tween test points for an initial experiment Smaller intervals may be appropriate when a concentration-response is being investigated

(*3*) Testing above the concentration of 5 mg/plate or 5µl/plate may be considered when evaluating substances containing substantial amounts of potentially mutagenic impurities

(C) **Controls.** (*1*) Concurrent strain-specific positive and negative (solvent or vehicle) controls, both with and without metabolic activation, should be included in each assay Positive control concentrations that demonstrate the effective performance of each assay should be selected

(*2*) For assays employing a metabolic activation system, the positive control reference substance(s) should be selected on the basis of the type of bacteria strains used The following chemicals are examples of suitable positive controls for assays with metabolic activation:

Chemical	CAS number
9,10-Dimethylanthracene	[781–43–1]
7,12-Dimethylbenzanthracene	[57–97–6]
Congo Red (for the reductive metabolic activation method)	[573–58–0]
Benzo(a)pyrene	[50–32–8]
Cyclophosphamide (monohydrate)	[50-18-0] [6055–19–2]
2-Aminoanthracene	[613–13–8]

2–Aminoanthracene should not be used as the sole indicator of the efficacy of the S9–mix. If 2–aminoanthracene is used, each batch of S9 should also be characterised with a mutagen that requires metabolic activation by microsomal enzymes, e g , benzo(a)pyrene, dimethylbenzanthracene.

(*3*) For assays performed without metabolic activation system, examples of strain-specific positive controls are:

al	CAS number	Strain
(a) Sodium azide	[26628–22–8]	TA1535 and TA100
(b) 2-Nitrofluorene	[607–57–8]	TA 98
(c) 9-Aminoacridine or ICR 191	[90–45–9] [17070–45–0]	TA1537, TA97 and TA97a
(d) Cumene hydroperoxide	[80–15–9]	TA102
(e) Mitomycin C	[50–07–7]	WP2 *uvrA* and TA102
(f) N-Ethyl-N-nitro-N-nitrosoguanidine or 4-nitroquinoline 1-oxide	[70–25–7] [56–57–5]	WP2, WP2 *uvrA* and WP2 *uvrA* (pKM101)
(g) Furylfuramide (AF-2)	[3688–53–7]	Plasmid-containing strains

(*4*) Other appropriate positive control reference substances may be used The use of chemical class-related positive control chemicals may be considered, when available

(5) Negative controls, consisting of solvent or vehicle alone, without test substance, and otherwise treated in the same way as the treatment groups, should be included In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent

(3) **Procedure**—(i) **Treatment with test substance.** (A) For the plate incorporation method (see references in paragraphs (g)(1), (g)(2), (g)(3), and (g)(4) of this guideline), without metabolic activation, usually 0 05 ml or 0 1 ml of the test solutions, 0 1 ml of fresh bacterial culture (containing approximately 10^8 viable cells) and 0 5 ml of sterile buffer are mixed with 2 0 ml of overlay agar For the assay with metabolic activation, usually 0 5 ml of metabolic activation mixture containing an adequate amount of post-mitochondrial fraction (in the range from 5 to 30 percent v/v in the metabolic activation mixture) are mixed with the overlay agar (2 0 ml), together with the bacteria and test substance/test solution The contents of each tube are mixed and poured over the surface of a minimal agar plate The overlay agar is allowed to solidify before incubation

(B) For the preincubation method (see references in paragraphs (g)(2), (g)(3), (g)(5), and (g)(6) of this guideline) the test substance/test solution is preincubated with the test strain (containing approximately 10^8 viable cells) and sterile buffer or the metabolic activation system (0.5 ml) usually for 20 min or more at 30–37 °C prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate Usually, 0 05 or 0 1 ml of test substance/test solution, 0 1 ml of bacteria, and 0.5 ml of S9–mix or sterile buffer, are mixed with 2 0 ml of overlay agar Tubes should be aerated during pre-incubation by using a shaker.

(C) For an adequate estimate of variation, triplicate plating should be used at each dose level The use of duplicate plating is acceptable when scientifically justified The occasional loss of a plate does not necessarily invalidate the assay

(D) Gaseous or volatile substances should be tested by appropriate methods, such as in sealed vessels (see references in paragraphs (g)(12), (g)(14), (g)(15), and (g)(16) of this guideline)

(ii) **Incubation.** All plates in a given assay should be incubated at 37 °C for 48–72 hours After the incubation period, the number of revertant colonies per plate is counted

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be presented as the number of revertant colonies per plate. The number of revertant colonies on both negative (solvent control, and untreated control if used) and positive control plates should also be given

(ii) Individual plate counts the mean number of revertant colonies per plate and the standard deviation should be presented for the test substance and positive and negative (untreated and/or solvent) controls

(iii) There is no requirement for verification of a clear positive response Equivocal results should be clarified by further testing preferably using a modification of experimental conditions Negative results need to be confirmed on a case-by-case basis In those cases where confirmation of negative results is not considered necessary, justification should be provided Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments Study parameters that might be modified include the concentration spacing, the method of treatment (plate incorporation or liquid preincubation), and metabolic activation conditions

(2) **Evaluation and interpretation of results.** (i) There are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system (see reference in paragraph (g)(23) of this guideline) Biological relevance of the results should be considered first Statistical methods may be used as an aid in evaluating the test results (see reference in paragraph (g)(24) of this guideline) However, statistical significance should not be the only determining factor for a positive response

(ii) A test substance for which the results do not meet the above criteria is considered non-mutagenic in this test

(iii) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance Results may remain equivocal or questionable regardless of the number of times the experiment is repeated

(iv) Positive results from the bacterial reverse mutation test indicate that a test substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli* Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

(3) **Test report.** The test report should include the following information.

(i) Test substance.

(A) Identification data and CAS no , if known

(B) Physical nature and purity

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known

(ii) Solvent/vehicle

(A) Justification for choice of solvent/vehicle

(B) Solubility and stability of the test substance in solvent/vehicle, if known

(iii) Strains

(A) Strains used

(B) Number of cells per culture

(C) Strain characteristics

(iv) Test conditions

(A) Amount of test substance per plate (mg/plate or ml/plate) with rationale for selection of dose and number of plates per concentration

(B) Media used

(C) Type and composition of metabolic activation system, including acceptability criteria

(D) Treatment procedures

(v) Results

(A) Signs of toxicity

(B) Signs of precipitation

(C) Individual plate counts

(D) The mean number of revertant colonies per plate and standard deviation

(E) Dose-response relationship, where possible

(F) Statistical analyses, if any

(G) Concurrent negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations

(H) Historical negative (solvent/vehicle) and positive control data, with e g., ranges, means and standard deviations

(vi) Discussion of the results

(vii) Conclusion

(g) **References.** The following references should be consulted for additional background information on this test guideline

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United States
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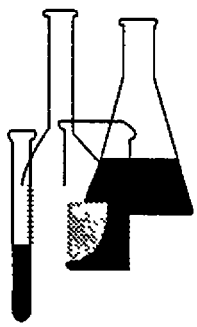
Health Effects Test Guidelines

OPPTS 870.5140
Gene Mutation in
Aspergillus nidulans



INTRODUCTION

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The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C. 136, *et seq*)

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OPPTS 870.5140 Gene mutation in Aspergillus nidulans

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136 *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5140 Gene mutations in Aspergillus nidulans and OPP 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982.

(b) **Purpose.** *Aspergillus nidulans* (*A nidulans*) is a eukaryotic fungus which has been developed to detect and study a variety of genetic phenomena including chemically induced mutagenesis *A nidulans* can be used to detect both forward and reverse gene mutation. These mutations are detected by changes in colonial morphology or nutritional requirements in treated populations The methionine and 2-thioxanthine forward mutation systems can be used to detect mutations in *A nidulans*

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definition also applies to this test guideline.

Forward mutation is a gene mutation from the wild (parent) type to the mutant condition

(d) **Reference substances.** These may include, but need not be limited to, ethyl methanesulfonate, cyclophosphamide, or aflatoxin B_1

(e) **Test method**—(1) **Principle.** Conidia are exposed to the test chemical both with and without metabolic activation and plated on selective medium to determine changes in colonial morphology or nutritional requirements At the end of a suitable incubation period, mutant colonies are counted and compared to the number of spontaneous mutants in an untreated control culture Simultaneous determination of survival permits calculation of mutation frequency

(2) **Description.** Tests for mutation in *A nidulans* are performed in liquid suspension Treated conidia are plated on selective medium to determine changes in nutritional requirements or colonial morphology.

(3) **Strain selection**—(i) **Designation.** For the methionine and 2-thioxanthine systems the haploid Glascow *biAl, meth G1* strain is the most commonly used strain although other strains may be appropriate Any translocation-free strain which produces green colonies on thioxanthine free medium and yellow colonies on medium containing thioxanthine may be used in the thioxanthine system

(ii) **Preparation and storage** Stock culture preparation and storage growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented

(iii) **Media.** Any medium which supports growth and a characteristic colonial morphology may be used in the assay

(4) **Preparation of conidia.** Prior to chemical treatment, conidia from four to five single colonies of the appropriate strain are grown at 37 °C on complete medium At the end of the incubation period, conidia are collected, conidial chains broken up, mycelial debris removed and conidia concentrated prior to removal of the germination inhibitory substance Germination inhibitory substance should be removed by Tween 80 or diethyl ether

(5) **Metabolic activation.** Conidia should be exposed to a test substance both in the presence and absence of an appropriate metabolic activation system

(6) **Control groups.** Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment

(7) **Test chemicals—(i) Vehicle.** Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) **Exposure concentrations.** (A) The test should initially be performed over a broad range of concentrations selected on the basis of a preliminary assay. Effective treatment times should also be selected in the preliminary assay.

(B) Each test should include five treatment points, two at fixed concentrations for different time periods, and three at varying concentrations for fixed periods of time

(C) Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility Cytotoxicity of the test chemical may be altered in the presence of a metabolic activation system Relatively insoluble chemicals should be tested up to the limits of solubility For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis

(D) When appropriate, a positive response should be confirmed by using a narrow range of test concentrations

(f) **Test Performance—(1) Treatment.** Germinating or quiescent conidia in liquid suspension should be exposed to the test chemical at

37 °C under conditions of yellow light and controlled pH and oxygen tension At the end of the exposure period, treatment should be terminated by repeated centrifugation and washing of the conidia or by dilution Chemical neutralization of the test agent may also be used but is not recommended

(2) **Media**—(i) **Methionine system.** For the methionine system, conidia should be plated on methionine deficient medium for mutant selection and on medium supplemented with methionine to determine survival

(ii) **Thioxanthine system.** (A) For the 2-thioxanthine system, treated conidia should be plated on nitrogen-free glucose and salts minimal medium containing 2-thioxanthine

(B) After incubation, green colonies should be counted and isolated by restreaking The isolated colonies should be classified on the basis of genetic criteria Yellow, wild-type colonies will grow on the same plate This permits concurrent determination of survival and an estimation of mutation frequency

(3) **Determination of mutation frequency and viability.** In both systems, mutation frequency and viability should be determined immediately before and immediately after chemical treatment

(4) **Incubation conditions.** All incubations should be at 37 °C Incubation time will vary depending upon system and endpoint (mutation or viability) being determined

(5) **Number of cultures.** (i) At least 10 independent plates per concentration with no more than 20 colonies per plate should be used in the methionine system.

(ii) Fifteen to twenty plates per concentration are preferred for the 2-thioxanthine system

(g) **Data and report**—(1) **Treatment of results.** Individual plate counts for test substance and controls should be presented for both mutation induction and survival The mean number of colonies per plate and standard deviation should also be presented Data should be presented in tabular form indicating, as applicable, numbers of colonies counted, and numbers and classification of mutants identified Sufficient detail should be provided for verification of survival and mutation frequencies

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of mutant colonies. Another criterion may

be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of mutant colonies or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results from the methionine and 2-thioxanthine systems in *A nidulans* indicate that, under the test conditions, the test substance causes gene (point) mutations in the DNA of this organism caused by base-pair changes and small deletions in the genome

(ii) Negative results indicate that under the test conditions the test chemical is not mutagenic in *A nidulans*

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Strain of organism used in the assay

(ii) Test chemical vehicle, doses used, rationale for dose selection, and toxicity data

(iii) Method used for preparation of conidia

(iv) Treatment conditions, including length of exposure and method used to stop treatment.

(v) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay

(vi) Incubation times and temperature

(vii) Positive and negative controls

(viii) Dose-response relationship, if applicable

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Ames, B N et al Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutation Research* 31.347-364 (1975)

(2) Kafer, E et al *Aspergillus nidulans*· systems and results of tests for chemical induction of mitotic segregation and mutation. I Diploid and

duplication assay systems a report of the U S EPA s Gene-Tox Program *Mutation Research* 98 1–48 (1982)

(3) Munson, R J and Goodhead, D T Relation between induced mutation frequency and cell survival a theoretical approach and an examination of experimental data for eukaryotes *Mutation Research* 42 145–159 (1977)

(4) Scott, B R et al *Aspergillus nidulans* systems and results of tests for mitotic segregation and mutation II Haploid assay systems and overall response of all systems a report of the U S EPA's Gene-Tox Program. *Mutation Research* 98 49–94 (1982)

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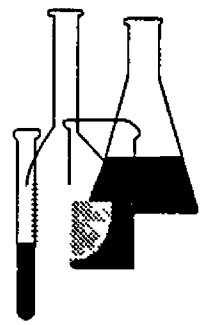
Health Effects Test Guidelines

OPPTS 870.5195
Mouse Biochemical Specific Locus Test



INTRODUCTION

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OPPTS 870 5195 Mouse biochemical specific locus test.

(a) **Scope**—(1) **Applicability** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798.5195 Mouse biochemical specific locus test and OPP 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982.

(b) **Purpose.** The mouse biochemical specific locus test (MBSL) may be used to detect and quantitate mutations originating in the germ line of a mammalian species

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Biochemical specific locus mutation is a genetic change resulting from a DNA lesion causing alterations in proteins that can be detected by electrophoretic methods

Germ line comprises the cells in the gonads of higher eukaryotes, which are the carriers of the genetic information for the species.

(d) **Test method**—(1) **Principle.** The principle of the MBSL is that heritable damage to the genome can be detected by electrophoretic analysis of proteins in the tissues of the progeny of mice treated with germ cell mutagens

(2) **Description.** For technical reasons, males rather than females are generally treated with the test chemical Treated males are then mated to untreated females to produce F_1 progeny Both blood and kidney samples are taken from progeny for electrophoretic analysis. Up to 33 loci can be examined by starch-gel electrophoresis and broad-range isoelectric focussing. Mutants are identified by variations from the normal electrophoretic pattern Presumed mutants are bred to confirm the genetic nature of the change

(3) **Animal selection**—(i) **Species and strain.** Mice should be used as the test species Although the biochemical specific locus test could be performed in a number of inbred strains, in the most frequently used cross, C57BL/6 females are mated to DBA/2 males to produce (C57BL/6 × DBA/2) F_1 progeny

(ii) **Age.** Healthy, sexually mature (at least 8 weeks old) animals should be used for treatment and breeding

(iii) **Number.** A decision on the minimum number of treated animals should take into account possible effects of the test chemical on the fertility of the treated animals Other considerations should include

(A) The production of concurrent spontaneous controls

(B) The use of positive controls

(C) The power of the test

(4) **Control groups**—(i) **Concurrent controls.** An appropriate number of concurrent control loci should be analyzed in each experiment These should be partly derived from matings of untreated animals (from 5 to 20 percent of the treated matings), although some data on control loci can be taken from the study of the alleles transmitted from the untreated parent in the experimental cross However, any laboratory which has had no prior experience with the test should produce a spontaneous control sample of about 5,000 progeny animals and a positive control sample (using 100 mg/kg ethylnitrosourea) of at least 1,200 offspring

(ii) **Historical controls.** Long-term, accumulated spontaneous control data (currently, 1 mutation in 1,200,000 control loci screened) are available for comparative purposes

(5) **Test chemicals**—(i) **Vehicle.** When possible, test chemicals should be dissolved or suspended in distilled water or buffered isotonic saline Water-insoluble chemicals should be dissolved or suspended in appropriate vehicles The vehicle used should neither interfere with the test chemical nor produce major toxic effects Fresh preparations of the test chemical should be employed

(ii) **Dose levels** Usually, only one dose need be tested. This should be the maximum tolerated dose (MTD), the highest dose tolerated without toxic effects. Any temporary sterility induced due to elimination of spermatogonia at this dose must be of only moderate duration, as determined by a return of males to fertility within 80 days after treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) **Route of administration.** Acceptable routes of administration include, but are not limited to, gavage, inhalation, and mixture with food or water, and intraperitoneal or intravenous injections

(e) **Test performance**—(1) **Treatment and mating.** Male DBA/2 mice should be treated with the test chemical and mated to virgin C57BL/6 females immediately after cessation of treatment Each treated male should be mated to new virgin C57BL/6 females each week Each pairing will continue for a week until the next week's mating is to begin This mating schedule permits sampling of all post-spermatogonial stages of germ-cell development during the first 7 weeks after exposure

Spermatogonial stem cells are studied thereafter Repeated mating cycles should be conducted until sufficient offspring have been obtained to meet the power criterion of the assay for spermatogonial stem cells

(2) **Examination of offspring**—(i) **Birth and weaning.** Offspring should be examined at birth and at weaning for externally detectable changes in morphology and behavior, these could be due to dominant mutations Such characteristics may include, but are not limited to, variations in coat color, appearance of eyes, size (in which case weighing of variant animals and littermates should be carried out), fur texture, etc Gross changes in external form and behavior should also be sought Scrutiny of such visible characteristics of all animals should be made during all subsequent manipulations of the animals

(ii) **Tissue sampling** Blood (about 0 1 mL) and one kidney should be removed from progeny mice under anesthesia Both tissues are then prepared for analysis by electrophoresis

(iii) **Electrophoresis** The gene products of 6 loci should be analyzed in the blood sample by broad-range isoelectric focusing and of 27 loci in the kidney sample by starch-gel electrophoresis and enzyme-specific staining Details on these procedures are included in paragraphs (h)(2) and (h)(3) of this guideline

(iv) **Mutant identification** Presumptive electrophoretic mutants should be identified by variation from the normal electrophoretic banding patterns Reruns of all variant samples should be performed to confirm the presence of altered banding patterns Samples from parents of progeny exhibiting banding pattern variations should be assayed to determine whether the variant was induced by the experimental treatment or was pre-existing. All treatment-induced variants are bred to determine the genetic nature of the change.

(f) **Data and reports**—(1) **Treatment of results.** Data should be presented in tabular form and should permit independent analysis of cell stage-specific effects, and dose-dependent phenomena The data should be recorded and analyzed in such a way that clusters of identical mutations are clearly identified The individual mutants detected should be thoroughly described In addition, concurrent positive control data (if employed) and spontaneous control data should also be tabulated These concurrent controls should be added to, as well as compared with, the historical control data

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results** (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the frequency of electrophoretic mutations Another cri-

terion may be based upon detection of a reproducible and statistically significant positive response for at least one of these test points

(ii) A test chemical which does not produce a statistically significant increase in the frequency of electrophoretic mutations over the spontaneous frequency, or a statistically significant and reproducible positive response for at least one of the test points, is considered nonmutagenic in this system, provided that the sample size is sufficient to exclude a biologically significant increase in mutation frequency

(iii) Biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation** (i) Positive results in the MBSL indicate that, under the test conditions, the test chemical induces heritable gene mutations in a mammalian species

(ii) Negative results indicate that, under the test conditions, the test chemical does not induce heritable gene mutations in a mammalian species

(5) **Test report** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) Strain, age, and weight of animals used, numbers of animals of each sex in experimental and control groups

(ii) Test chemical vehicle, doses used, rationale for dose selection, and toxicity data, if available

(iii) Route and duration of exposure

(iv) Mating schedule

(v) Number of loci screened for both treated and spontaneous data

(vi) Criteria for scoring mutants

(vii) Number of mutants found/locus

(viii) Loci at which mutations were found

(ix) Use of concurrent negative and positive controls

(x) Dose-response relationship, if applicable

(g) **Additional requirements.** Testing facilities conducting the mouse biochemical specific locus test in accordance with this section should, in addition to adhering to the provisions of 40 CFR 792 190 and 792 195, obtain, adequately identify and retain for at least 10 years, acceptable 35–mm photographs (and their negatives) of the stained isoelectric-focusing

columns and the stained starch-gels obtained following analyses of blood and kidney preparations respectively, from mutant mice, their siblings, and their parents

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Johnson, F M et al The detection of mutants in mice by electrophoresis Results of a model induction experiment with procarbazine *Genetics* 97 113–124 (1981)

(2) Johnson, F M and Lewis, S E Mutation rate determinations based on electrophoretic analysis of laboratory mice *Mutation Research* 82 125–135 (1981)

(3) Johnson, F M and Lewis, S E Electrophoretically detected germinal mutations induced by ethylnitrosourea in the mouse *Proceedings of the National Academy of Sciences* 78 3138–3141 (1981)

(4) Lewis, S E et al Dominant visible and electrophoretically expressed mutations induced in male mice exposed to ethylene oxide by inhalation *Environmental Mutagenesis* 8 867–872 (1986)

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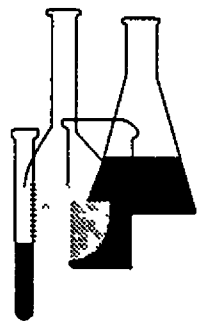
Health Effects Test Guidelines

OPPTS 870.5200
Mouse Visible Specific Locus Test



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OPPTS 870 5200 Mouse visible specific locus test

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136 *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5200 Mouse visible specific locus test and OPP 84-2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982

(b) **Purpose.** The mouse visible specific locus test (MSLT) may be used to detect and quantitate mutations in the germ line of a mammalian species

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Germ line is the cells in the gonads of higher eukaryotes which are the carriers of the genetic information for the species

Visible specific locus mutation is a genetic change that alters factors responsible for coat color and other visible characteristics of certain mouse strains

(d) **Test method**—(1) **Principle.** (i) The principle of the MSLT is to cross individuals who differ with respect to the genes present at certain specific loci, so that a genetic alteration involving the standard gene at any one of these loci will produce an offspring detectably different from the standard heterozygote The genetic change may be detectable by various means, depending on the loci chosen to be marked

(ii) Three variations of the method currently exist for detecting newly arising point mutations in mouse germ cells

(A) The visible specific locus test using either five or seven loci.

(B) The biochemical specific locus test using up to 20 enzymes

(C) The test for mutations at histocompatibility loci

(iii) Of the three tests, the visible specific locus test has been most widely used in assessing genetic hazard due to environmental agents. It is the method described in this guideline

(2) **Description.** For technical reasons, males rather than females are generally treated with the test agent Treated males are then mated to females which are genetically homozygous for certain specific visible marker

loci Offspring are examined in the next generation for evidence that a new mutation has arisen

(3) **Animal selection**—(i) **Species and strain.** Mice should be used as the test species Male mice should be either $(C_3H \times 101)F_1$ or $(101 \times C_3H)F_1$ hybrids Females should be T stock virgins

(ii) **Age.** Healthy sexually mature animals should be used

(iii) **Number.** A decision on the minimum number of treated animals should take into account the spontaneous variation of the biological characterization being evaluated Other considerations should include

(A) The use of either historical or concurrent controls

(B) The power of the test

(C) The minimal rate of induction required

(D) The use of positive controls

(E) The level of significance desired

(iv) **Assignment to groups.** Animals should be randomized and assigned to treatment and control groups

(4) **Control groups**—(i) **Concurrent controls.** The use of positive or spontaneous controls is left to the discretion of the investigator. However, any laboratory which has had no prior experience with the test should, at its first attempt, produce a negative control sample of 20,000 and a positive control, using 100 mg/kg 1-ethylnitrosourea, in a sample of 5,000 offspring

(ii) **Historical controls.** Long-term, accumulated spontaneous control data of 43/801,406 are available for comparative purposes

(5) **Test chemicals**—(i) **Vehicle.** When possible, test chemicals should be dissolved or suspended in distilled water or isotonic saline buffered appropriately, if needed, for stability. Water-insoluble chemicals should be dissolved or suspended in appropriate vehicles The vehicle used should neither interfere with the test compound nor produce major toxic effects Fresh preparations of the test chemical should be employed

(ii) **Dose levels.** Usually, only one-dose level need be tested This should be the highest dose tolerated without toxic effects, provided that any temporary sterility induced due to elimination of spermatagonia is of only moderate duration, as determined by a return of males to fertility within 80 days after treatment For evaluation of dose-response, it is recommended that at least two dose levels be tested

(iii) **Route of administration.** Acceptable routes of administration include gavage, inhalation, admixture with food or water, and IP or IV injections

(e) **Test performance—(1) Treatment and mating** Hybrid F_1 ($C_3H \times 101$) or ($101 \times C_3H$) male mice should be treated with the test substance and immediately mated to virgin T stock females Each treated male should be mated to a fresh group of two to four virgin females each week for 7 weeks, after which he should be returned to the first group of females and rotated through the seven sets of females repeatedly This mating schedule generally permits sampling of all postspermatagonial stages of germ cell development during the first 7 weeks and rapid accumulation of data for exposed spermatagonial stem cells thereafter Repeated mating cycles should be conducted until the entire spermatogonial cycle has been evaluated and enough offspring have been obtained to meet the power criterion of the assay

(2) **Examination of offspring.** (i) Offspring may be examined at (or soon after) birth but must be examined at about 3 weeks of age at which time the numbers of mutant and nonmutant offspring in each litter should be recorded

(ii) Nonmutant progeny should be discarded Mutant progeny should be subjected to genetic tests for verification

(f) **Data and report—(1) Treatment of results.** Data should be presented in tabular form and should permit independent analysis of cell-stage specific effects and dose-dependent phenomena. The data should be recorded and analyzed in such a way that clusters of identical mutations are clearly identified The individual mutants detected should be thoroughly described. In addition, concurrent positive and negative control data, if they are available, should be tabulated so that it is possible to differentiate between concurrent (when available) and long-term accumulated mutation frequencies

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of specific locus mutations Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of specific locus mutations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results in the MSLT indicate that under the test conditions the test substance induces heritable gene mutations in the test species

(ii) Negative results indicate that under the test conditions the test substance does not induce heritable gene mutations in the test species

(5) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Strain, age, and weight of animals used, number of animals of each sex in experimental and control groups

(ii) Test chemical vehicle, doses used, rationale for dose selection, and toxicity data

(iii) Route and duration of exposure

(iv) Mating schedule

(v) Time of examination for mutant progeny

(vi) Criteria for scoring mutants

(vii) Use of concurrent or negative controls

(viii) Dose response relationship, if applicable

(g) **Additional requirements.** Testing facilities conducting the mouse visible specific locus test in accordance with this section should, in addition to adhering to the provisions of 40 CFR 792 190 and 792.195 obtain, and retain for at least 10 years, acceptable 35–mm color photographs (and their negatives) demonstrating the visible mutations observed in mutant animals and the lack of such mutations in their siblings and parents

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Russell, L.B et al. The mouse specific locus test with agents other than radiations interpretation of data and recommendations for future work A report of the U S EPA's Gene-Tox Program *Mutation Research* 86.329–354 (1981)

(2) [Reserved]

United States
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Prevention Pesticides
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(7101)

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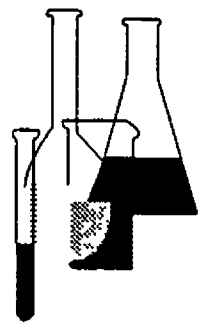
Health Effects Test Guidelines

OPPTS 870.5250
Gene Mutation in
Neurospora crassa



INTRODUCTION

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The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.5250 Gene mutation in Neurospora crassa

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5250 Gene mutation in Neurospora crassa and OPP 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982

(b) **Purpose** *Neurospora crassa* (*N crassa*) is a eukaryotic fungus which has been developed to detect and study a variety of genetic phenomena including chemically induced mutagenesis *N crassa* can be used to detect both forward and reverse gene mutation These mutations are detected by biochemical or morphological changes in the treated population The most commonly used mutation assay in *N crassa* measures forward mutation in the *ad-3* region of the genome

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definition also applies to this test guideline

Forward mutation is a gene mutation from the wild (parent) type to the mutant condition

(d) **Reference substances.** These may include, but need not be limited to, ethyl or methyl methanesulfonate

(e) **Test method**—(1) **Principle.** The detection of forward mutations at the *ad-3* locus in either homokaryons or heterokaryons may be used However, use of two component heterokaryons is recommended because of the greater range of mutations which can be recovered In either case, the test relies on the identification of purple (mutant) colonies among a large number of white (wild-type) colonies A representative sample of purple colonies can be recovered and thoroughly analyzed genetically.

(2) **Description.** Forward mutations at the *ad-3* locus can be detected using noncolonial strains of *N crassa* grown on media containing sorbose as well as glucose. Under these conditions, colonies are formed and reproducible colonial morphology results Adenine-requiring mutants which accumulate a reddish-purple pigment can be readily identified and counted

(3) **Strain selection**—(i) **Designation.** At the present time, heterokaryon 12 is recommended for use in this assay The use of other strains may also be appropriate

(ii) **Preparation and storage.** Stock culture preparation and storage, growth requirements, method of strain identification, and demonstration

of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented

(iii) **Media.** Frie's No 3 minimal medium or Westgaard's Synthetic medium with 1 5 percent agar or any medium known to support growth and characteristic colonial morphology may be used in the assay

(4) **Preparation of conidia.** Stock cultures should be grown on minimal medium to select for single colonies with noncolonial morphology Single-colony isolates then should be inoculated into agar flasks and incubated at 35 °C for 48 hours to select colonies with spreading growth patterns in which mycelia cover the entire flask Flasks should be incubated at 23-25°C and those with bright orange conidia selected for preparation of conidial suspensions Suspensions should be diluted for use in distilled water

(5) **Metabolic activation.** Conidia should be exposed to a test substance both in the presence and absence of an appropriate metabolic activation system.

(6) **Control groups.** Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment

(7) **Test chemicals—(i) Vehicle.** Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay

(ii) **Exposure concentrations.** (A) The test should initially be performed over a broad range of concentrations selected on the basis of a preliminary assay Effective treatment times should also be selected in the preliminary assay.

(B) Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems For toxic chemicals, the highest concentration tested should not reduce survival below 10 percent of that seen in the control cultures Relatively insoluble chemicals should be tested up to the limits of solubility For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis.

(C) Each test should include five treatment points, two at fixed concentrations for different time periods and three at varying concentrations for fixed periods of time

(D) When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations

(f) **Test performance**—(1) **Treatment.** (i) Growing or nongrowing conidia should be exposed to the test chemical with and without metabolic activation At the end of the exposure period, treatment should be terminated by chemical quenching The quenching solution may contain 0 1 percent sodium thiosulfate

(ii) Conidia should then be plated on the appropriate media to determine mutation induction and viability At the end of the incubation period, colonies should be scored for viability and mutation induction

(iii) Mutants should be classified according to color and morphology

(iv) Both mutation frequency and viability should be determined both immediately before and immediately after chemical treatment

(2) **Incubation conditions.** All plates in a given test should be incubated for the same time period This incubation period may be from 2 to 7 days at 30°C

(3) **Number of cultures.** Generally, 15 to 20 individual plates per concentration should be used

(g) **Data and report**—(1) **Treatment of results.** Individual plate counts for test substance and controls should be presented for both mutation induction and survival The mean number of colonies per plate and standard deviation should be presented Data should be presented in tabular form indicating, as applicable, numbers of colonies counted, numbers of mutants identified, and classification of mutants (e.g , color segregants). Sufficient detail should be provided for verification of survival and mutation frequencies

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical techniques

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of mutant colonies Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of mutant colonies or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results from the *ad-3* system in *N crassa* indicate that, under the test conditions, the test substance causes mutations in the DNA of this organism

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *N crassa*

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Strain of organism used in the assay

(ii) Test chemical vehicle, doses used, and rationale for dose selection

(iii) Method used for preparation of conidia

(iv) Treatment conditions, including length of exposure and method used to stop treatment

(v) Incubation times and temperature

(vi) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay

(vii) Dose-response relationship, if applicable

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Brockman, H.E. and de Serres, F J. Induction of *ad-3* mutants of *Neurospora crassa* by 2-aminopurine *Genetics* 48· 597–604 (1963)

(2) de Serres, F.J and Malling, H V Measurement of recessive lethal damage over the entire genome and at two specific loci in the ad-3 region of a two-component heterokaryon of *Neurospora crassa* Chemical mutagens: principles and methods for their detection. Vol 2, Ed Hollaender, A Plenum, New York and London (1971) pp 311–342

(3) Matzinger, P.K and Ong, T–M In vitro activation of aflatoxin B_1 to metabolites mutagenic in *Neurospora crassa. Mutation Research* 37:27–32 (1976)

United States
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Prevention Pesticides
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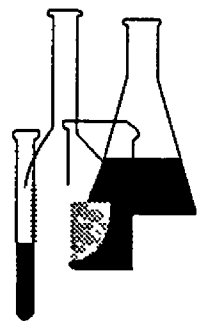
Health Effects Test Guidelines

OPPTS 870.5275 Sex-Linked Recessive Lethal Test in *Drosophila melanogaster*



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OPPTS 870.5275 Sex-linked recessive lethal test in Drosophila melanogaster.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5275 Sex-linked recessive lethal test in Drosophila melanogaster and OECD 477 Genetic Toxicology Sex-Linked Recessive Lethal Test in Drosophila melanogaster

(b) **Purpose.** The sex-linked recessive lethal (SLRL) test using *Drosophila melanogaster* (*D melanogaster*) detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome. This represents about 80 percent of all X-chromosome loci The X-chromosome represents approximately one-fifth of the entire haploid genome

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Lethal mutation is a change in the genome which, when expressed, causes death to the carrier.

Recessive mutation is a change in the genome which is expressed in the homozygous or hemizygous condition

Sex-linked genes are present on the sex (X or Y) chromosomes. Sex-linked genes in the context of this guideline refer only to those located on the X-chromosome

(d) **Reference substances.** These may include, but need not be limited to, ethyl methanesulfonate or *N*-nitrosodimethylamine

(e) **Test method**—(1) **Principle.** Mutations in the X-chromosome of *D melanogaster* are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes

(2) **Description.** Wild-type males are treated and mated to appropriate females Female offspring are mated individually to their brothers, and in the next generation the progeny from each separate dose are scored for phenotypically wild-type males Absence of these males indicates that

a sex-linked recessive lethal mutation has occurred in a germ cell of the P_1 male

(3) **Drosophila stocks.** Males of a well-defined wild type stock and females of the Muller-5 stock may be used Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used

(4) **Control groups—(i) Concurrent controls.** Concurrent positive and negative (vehicle) controls should be included in each experiment

(ii) **Positive controls.** Examples of positive controls include ethyl methanesulfonate and *N*-nitrosodimethylamine

(iii) **Other positive controls.** Other positive control reference substances may be used

(iv) **Negative controls.** Negative (vehicle) controls should be included The size of the negative (vehicle) control group should be determined by the availability of appropriate laboratory historical control data.

(5) **Test chemicals—(i) Vehicle.** Test chemicals should be dissolved in water Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e g , a mixture of ethanol and Tween-60 or 80) and then diluted in water or saline prior to administration The use of dimethylsulfoxide as a vehicle should be avoided

(ii) **Dose levels.** For the initial assessment of mutagenicity, it is sufficient to test a single dose of the test substance for screening purposes This dose should be the maximum tolerated dose, or that which produces some indication of toxicity, or should be the highest dose attainable. For dose-response purposes, at least three additional dose levels should be used

(iii) **Route of administration.** Exposure may be oral, by injection or by exposure to gases or vapors Feeding of the test compound may be done in sugar solution When necessary, substances may be dissolved in 0 7 percent NaCl solution and injected into the thorax or abdomen.

(f) **Test performance—(1) Treatment and mating.** Wild-type males (3 to 5 days old) should be treated with the test substance and mated individually to an appropriate number of virgin females from the Muller-5 stock or females from another appropriately marked (with multiply-inverted X-chromosomes) stock The females should be replaced with fresh virgins every 2 to 3 days to cover the entire germ cell cycle The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment

(2) **F_1 matings.** Heterozygous F_1 females from the above crosses should be allowed to mate individually (i e , one female per vial) with

their brothers In the F_2 generation, each culture should be scored for the absence of wild-type males If a culture appears to have arisen from an F_1 female carrying a lethal in the parental X-chromosome (i e , no males with the treated chromosome are observed), daughters of that female with the same genotype should be tested to ascertain if the lethality is repeated in the next generation

(3) **Number of matings.** (i) The test should be designed with a predetermined sensitivity and power The number of flies in each group should reflect these defined parameters The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analysed to detect substances which show mutation rates close to those of the controls.

(ii) Test results should be confirmed in a separate experiment

(g) **Data and report—(1) Treatment of results.** Data should be tabulated to show the number of chromosomes tested, the number of nonfertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated Numbers of clusters of different size per male should be reported

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical techniques

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of sex-lined recessive lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of sex-linked recessive lethals or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation** (i) Positive results in the SLRL test in *D melanogaster* indicate that under the test conditions the test agent causes mutations in germ cells of this insect

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *D melanogaster*

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information should be reported

(i) *Drosophila* stock used in the assay, age of insects, number of males treated, number of sterile males, number of F_2 cultures established, number of F_2 cultures without progeny

(ii) Test chemical vehicle, treatment and sampling schedule, exposure levels, toxicity data, negative (vehicle) and positive controls, if appropriate

(iii) Criteria for scoring lethals

(iv) Number of chromosomes tested, number of chromosomes scored, number of chromosomes carrying a lethal mutation

(v) Historical control data, if available

(vi) Dose-response relationship, if applicable

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Sobels, F.H and Vogel, E The capacity of *Drosophila* for detecting relevant genetic damage *Mutation Research* 41 95–106 (1976).

(2) Wurgler F E et al *Drosophila* as assay system for detecting genetic changes Handbook of mutagenicity test procedures. Eds. Kilbey, B.J , Legator, M , Nichols, W , Ramel, C Elsevier/North Holland Biomedical, Amsterdam (1977) pp 335–373

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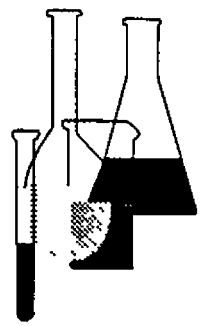
Health Effects Test Guidelines OPPTS 870.5300 *In Vitro* Mammalian Cell Gene Mutation Test



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OPPTS 870 5300 In vitro mammalian cell gene mutation test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5300 Detection of gene mutations in somatic cells in culture and OECD 476, *In Vitro* Mammalian Cell Gene Mutation Test

(b) **Introduction.** The *in vitro* mammalian cell gene mutation test can be used to detect gene mutations induced by chemical substances. Suitable cell lines include L5178Y mouse lymphoma cells, the CHO, AS52 and V79 lines of Chinese hamster cells, and TK6 human lymphoblastoid cells (see reference in paragraph (g)(1) of this guideline) In these cell lines the most commonly-used genetic endpoints measure mutation at thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), and a transgene of xanthine-guanine phosphoribosyl transferase (XPRT) The TK, HPRT and XPRT mutation tests detect different spectra of genetic events The autosomal location of TK and XPRT may allow the detection of genetic events (e g , large deletions) not detected at the HPRT locus on X-chromosomes (see references in paragraphs (g)(2), (g)(3), (g)(4),(g)(5), and (g)(6) of this guideline)

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Base pair substitution mutagens are substances which cause substitution of one or several base pairs in the DNA

Forward mutation is a gene mutation from the parental type to the mutant form which gives rise to an alteration or a loss of the enzymatic activity or the function of the encoded protein

Frameshift mutagens are substances which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

Mutant frequency is the number of mutant cells observed divided by the number of viable cells

Phenotypic expression time is a period during which unaltered gene products are depleted from newly mutated cells

Relative suspension growth is an increase in cell number over the expression period relative to the negative control

Relative total growth is an increase in cell number over time compared to a control population of cells, calculated as the product of suspen-

sion growth relative to the negative control times cloning efficiency relative to negative control

Survival is the cloning efficiency of the treated cells when plated at the end of the treatment period, survival is usually expressed in relation to the survival of the control cell population

Viability is the cloning efficiency of the treated cells at the time of plating in selective conditions after the expression period

(d) **Initial considerations.** (1) In the *in vitro* mammalian cell gene mutation test, cultures of established cell lines or cell strains can be used The cells used are selected on the basis of growth ability in culture and stability of the spontaneous mutation frequency Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions Care should be taken to avoid conditions which would lead to results not reflecting intrinsic mutagenicity Positive results which do not reflect intrinsic mutagenicity may arise from changes in pH, osmolality or high levels of cytotoxicity (see reference in paragraph (g)(7) of this guideline)

(2) This test is used to screen for possible mammalian mutagens and carcinogens Many compounds that are positive in this test are mammalian carcinogens, however, there is not a perfect correlation between this test and carcinogenicity Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through other, non-genotoxic mechanisms or mechanisms absent in bacterial cells (see reference in paragraph (g)(6) of this guideline).

(e) **Test method**—(1) **Principle.** (i) Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-} \rightarrow TK^{-/-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT) Thymidine kinase proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not. Similarly, cells deficient in HPRT or XPRT are selected by resistance to 6-thioguanine (TG) or 8-azaguanine (AG) The properties of the test substance should be considered carefully if a base analogue or a compound related to the selective agent is tested in any of the mammalian cell gene mutation tests. For example, any suspected selective toxicity by the test substance for mutant and non-mutant cells should be investigated. Thus, performance of the selection system/agent must be confirmed when testing chemicals structurally related to the selective agent (see reference in paragraph (g)(8) of this guideline).

(ii) Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a suitable period

of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection (see references in paragraphs (g)(9), (g)(10), (g)(11), (g)(12), and (g)(13) of this guideline) Cytotoxicity is usually determined by measuring the relative cloning efficiency (survival) or relative total growth of the cultures after the treatment period The treated cultures are maintained in growth medium for a sufficient period of time, characteristic of each selected locus and cell type, to allow near-optimal phenotypic expression of induced mutations Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency (viability) After a suitable incubation time, colonies are counted The mutant frequency is derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium

(2) **Description**—(i) **Preparations**—(A) **Cells.** (*1*) A variety of cell types are available for use in this test including subclones of L5178Y, CHO, CHO-AS52, V79, or TK6 cells Cell types used in this test should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a stable spontaneous mutant frequency Cells should be checked for *Mycoplasma* contamination and should not be used if contaminated

(*2*) The test should be designed to have a predetermined sensitivity and power The number of cells, cultures and concentrations of test substance used should reflect these defined parameters (see reference in paragraph (g)(14) of this guideline) The minimal number of viable cells surviving treatment and used at each stage in the test should be based on the spontaneous mutation frequency A general guide is to use a cell number which is at least ten times the inverse of the spontaneous mutation frequency However, it is recommended to utilise at least 10^6 cells Adequate historical data on the cell system used should be available to indicate consistent performance of the test

(B) **Media and culture conditions.** Appropriate culture media and incubation conditions (culture vessels, temperature, CO_2 concentration and humidity) should be used Media should be chosen according to the selective systems and cell type used in the test. It is particularly important that culture conditions should be chosen that ensure optimal growth of cells during the expression period and colony forming ability of both mutant and non-mutant cells

(C) **Preparation of cultures.** Cells are propagated from stock cultures, seeded in culture medium and incubated at 37 °C. Prior to use in this test, cultures may need to be cleansed of pre-existing mutant cells.

(D) **Metabolic activation.** Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activa-

tion system The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (see references in paragraphs (g)(15), (g)(16), (g)(17), and (g)(18) of this guideline) or a combination of phenobarbitone and β-naphthoflavone (see references in paragraphs (g)(19) and (g)(20) of this guideline) The post-mitochondrial fraction is usually used at concentrations in the range from 1–10 percent v/v in the final test medium The choice and condition of a metabolic activation system may depend upon the class of chemical being tested In some cases it may be appropriate to utilize more than one concentration of post-mitochondrial fraction A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation The choice of the cell lines used should be scientifically justified (e g , by the relevance of the cytochrome P450 isoenzyme to the metabolism of the test substance)

(E) **Test substance/preparations.** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted if appropriate prior to treatment of the cells Liquid test substances may be added directly to the test systems and/or diluted prior to treatment. Fresh preparations should be employed unless stability data demonstrate the acceptability of storage

(ii) **Test conditions**—(A) **Solvent/vehicle.** The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity. If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first. When testing water-unstable substances, the organic solvents used should be free of water Water can be removed by adding a molecular sieve

(B) **Exposure concentrations.** (*1*) Among the criteria to be considered when determining the highest concentration are cytotoxicity and solubility in the test system and changes in pH or osmolality

(2) Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indicator of cell integrity and growth, such as relative cloning efficiency (survival) or relative total growth It may be useful to determine cytotoxicity and solubility in a preliminary experiment.

(*3*) At least four analysable concentrations should be used. Where there is cytotoxicity, these concentrations should cover a range from the maximum to little or no toxicity, this will usually mean that the concentration levels should be separated by no more than a factor between 2 and $\sqrt{10}$ If the maximum concentration is based on cytotoxicity then it should

result in approximately 10–20 percent but not less than 10 percent relative survival (relative cloning efficiency) or relative total growth For relatively non-cytotoxic compounds the maximum concentration should be 5 mg/ml, 5µl/ml, or 0 01 M, whichever is the lowest

(*4*) Relatively insoluble substances should be tested up to or beyond their limit of solubility under culture conditions Evidence of insolubility should be determined in the final treatment medium to which cells are exposed. It may be useful to assess solubility at the beginning and end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc Insolubility can be detected by using the unaided eye The precipitate should not interfere with the scoring

(C) **Controls.** (*1*) Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment When metabolic activation is used the positive control chemical should be one that requires activation to give a mutagenic response

(*2*) Examples of positive control substances include

Metabolic Activation condition	Locus	Chemical	CAS number
Absence of exogenous metabolic activation	HPRT	Ethylmethanesulfonate	[62–50–0]
		Ethylnitrosourea	[759–73–9]
	TK (small and large colonies)	Methylmethanesulfonate	[66–27–3]
	XPRT	Ethylmethanesulfonate	[62–50–0]
		Ethylnitrosourea	[759–73–9]
Presence of exogenous metabolic activation	HPRT	3-Methylcholanthrene	[56–49–5]
		N-Nitrosodimethylamine	[62–75–9]
		7,12-Dimethylbenzanthracene	[57–97–6]
	TK (small and large colonies)	Cyclophosphamide (monohydrate)	[50–18–0] ([6055–19–2])
		Benzo(a)pyrene	[50–32–8]
		3-Methylcholanthrene	[56–49–5]
	XPRT	N-Nitrosodimethylamine (for high levels of S-9)	[62–75–9]
		Benzo(a)pyrene	[50–32–8]

(*3*) Other appropriate positive control reference substances may be used, e g , if a laboratory has a historical data base on 5-Bromo 2′-deoxyuridine [CAS no 59–14–3], this reference substance could be used as well The use of chemical class-related positive control chemicals may be considered, when available

(*4*) Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment groups should be included In addition, untreated controls should also be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent

(3) **Procedure**—(i) **Treatment with test substance.** (A) Proliferating cells should be exposed to the test substance both with and without metabolic activation Exposure should be for a suitable period of time (usually 3 to 6 hours is effective) Exposure time may be extended over one or more cell cycles

(B) Either duplicate or single treated cultures may be used at each concentration tested When single cultures are used, the number of concentrations should be increased to ensure an adequate number of cultures for analysis (e g , at least eight analysable concentrations) Duplicate negative (solvent) control cultures should be used

(C) Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (see references in paragraphs (g)(21) and (g)(22) of this guideline)

(ii) **Measurement of survival, viability, and mutant frequency.** (A) At the end of the exposure period, cells should be washed and cultured to determine survival and to allow for expression of the mutant phenotype Measurement of cytotoxicity by determining the relative cloning efficiency (survival) or relative total growth of the cultures is usually initiated after the treatment period

(B) Each locus has a defined minimum time requirement to allow near optimal phenotypic expression of newly induced mutants (HPRT and XPRT require at least 6–8 days, and TK at least 2 days) Cells are grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency, respectively The measurement of viability (used to calculate mutant frequency) is initiated at the end of the expression time by plating in non-selective medium

(C) If the test substance is positive in the L5178Y $TK^{+/-}$-test, colony sizing should be performed on at least one of the test cultures (the highest positive concentration) and on the negative and positive controls If the test substance is negative in the L5178Y $TK^{+/-}$-test, colony sizing should be performed on the negative and positive controls In studies using $TK6TK^{+/-}$, colony sizing may also beperformed

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should include cytotoxicity and viability determination, colony counts and mutant frequencies for the treated and control cultures. In the case of a positive response in the L5178Y $TK^{+/-}$-test, colonies are scored using the criteria of small and large colonies on at least one concentration of the test substance (highest positive concentration) and on the negative and positive control. The molecular and cytogenetic nature of both large and small colony mutants has been explored in detail (see references in paragraphs (g)(23) and (g)(24) of this guideline) In the $TK^{+/-}$-test, colonies are scored using the criteria of normal growth (large) and slow growth (small) colonies (see reference in paragraph (g)(25) of this guideline) Mutant cells

that have suffered the most extensive genetic damage have prolonged doubling times and thus form small colonies This damage typically ranges in scale from the losses of the entire gene to karyotypically visible chromosome aberrations The induction of small colony mutants has been associated with chemicals that induce gross chromosome aberrations (see reference in paragraph (g)(26) of this guideline) Less seriously affected mutant cells grow at rates similar to the parental cells and form large colonies

(ii) Survival (relative cloning efficiencies) or relative total growth should be given Mutant frequency should be expressed as number of mutant cells per number of surviving cells

(iii) Individual culture data should be provided Additionally, all data should be summarized in tabular form

(iv) There is no requirement for verification of a clear positive response Equivocal results should be clarified by further testing preferably using a modification of experimental conditions Negative results need to be confirmed on a case-by-case basis In those cases where confirmation of negative results is not considered necessary, justification should be provided Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments for either equivocal or negative results Study parameters that might be modified include the concentration spacing, and the metabolic activation conditions.

(2) **Evaluation and interpretation of results.** (i) There are several criteria for determining a positive result, such as a concentration-related, or a reproducible increase in mutant frequency Biological relevance of the results should be considered first Statistical methods may be used as an aid in evaluating the test results Statistical significance should not be the only determining factor for a positive response

(ii) A test substance, for which the results do not meet the above criteria is considered non-mutagenic in this system.

(iii) Although most studies will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

(iv) Positive results for an *in vitro* mammalian cell gene mutation test indicate that the test substance induces gene mutations in the cultured mammalian cells used A positive concentration-response that is reproducible is most meaningful Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used

(3) **Test report.** The test report should include the following information

(i) Test substance

(A) Identification data and CAS no , if known

(B) Physical nature and purity

(C) Physicochemical properties relevant to the conduct of the study

(D) Stability of the test substance

(ii) Solvent/vehicle

(A) Justification for choice of vehicle/solvent

(B) Solubility and stability of the test substance in solvent/vehicle, if known

(iii) Cells.

(A) Type and source of cells

(B) Number of cell cultures

(C) Number of cell passages, if applicable.

(D) Methods for maintenance of cell cultures, if applicable

(E) Absence of *Mycoplasma*

(iv) Test conditions

(A) Rationale for selection of concentrations and number of cell cultures including e g , cytotoxicity data and solubility limitations, if available.

(B) Composition of media, CO_2 concentration.

(C) Concentration of test substance

(D) Volume of vehicle and test substance added

(E) Incubation temperature

(F) Incubation time

(G) Duration of treatment

(H) Cell density during treatment

(I) Type and composition of metabolic activation system including acceptability criteria

(J) Positive and negative controls

(K) Length of expression period (including number of cells seeded, and subcultures and feeding schedules, if appropriate)

(L) Selective agent(s)

(M) Criteria for considering tests as positive, negative or equivocal

(N) Methods used to enumerate numbers of viable and mutant cells

(O) Definition of colonies of which size and type are considered (including criteria for "small" and "large" colonies, as appropriate)

(v) Results

(A) Signs of toxicity

(B) Signs of precipitation

(C) Data on pH and osmolality during the exposure to the test substance, if determined

(D) Colony size if scored for at least negative and positive controls.

(E) Laboratory's adequacy to detect small colony mutants with the L5178Y $TK^{+/-}$ system, where appropriate

(F) Dose-response relationship, where possible.

(G) Statistical analyses, if any

(H) Concurrent negative (solvent/vehicle) and positive control data

(I) Historical negative (solvent/vehicle) and positive control data with ranges, means, and standard deviations.

(J) Mutant frequency

(vi) Discussion of the results

(vii) Conclusion

(g) **References.** The following references should be consulted for additional background information on this test guideline

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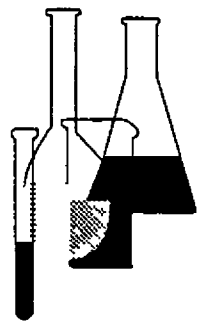
In Vitro Mammalian Chromosome Aberration Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.5375 In vitro mammalian chromosome aberration test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5375 In vitro mammalian cytogenetics and OECD 473, In Vitro Mammalian Chromosome Aberration Test

(b) **Purpose** (1) The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells (see paragraphs (i)(1), (i)(2), and (i)(3) of this guideline) Structural aberrations may be of two types, chromosome or chromatid With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations However, this guideline is not designed to measure numerical aberrations and is not routinely used for that purpose Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals

(2) The *in vitro* chromosome aberration test may employ cultures of established cell lines, cell strains or primary cell cultures. The cells used are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity, and spontaneous frequency of chromosome aberrations

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline.

Chromatid-type aberration is structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids

Chromosome-type aberration is structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site

Endoreduplication is a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period The result is chromosomes with 4, 8, 16, chromatids

Gap is an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatid(s)

Mitotic index is the ratio of cells in metaphase divided by the total number of cells observed in a population of cells, an indication of the degree of proliferation of that population

Numerical aberration is a change in the number of chromosomes from the normal number characteristic of the cells utilized

Polyploidy is a multiple of the haploid chromosome number (n) other than the diploid number (i e , 3n, 4n, and so on)

Structural aberration is a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges, and interchanges

(d) **Initial considerations** (1) Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation This metabolic activation system cannot mimic entirely the mammalian *in vivo* conditions Care should be taken to avoid conditions which would lead to positive results which do not reflect intrinsic mutagenicity and may arise from changes in pH, osmolality, or high levels of cytotoxicity (see paragraphs (i)(4) and (i)(5) of this guideline)

(2) This test is used to screen for possible mammalian mutagens and carcinogens Many compounds that are positive in this test are mammalian carcinogens, however, there is not a perfect correlation between this test and carcinogenicity. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage

(e) **Principle of the test method**. Cell cultures are exposed to the test substance both with and without metabolic activation At predetermined intervals after exposure of cell cultures to the test substance, they are treated with a metaphase-arresting substance (e.g., Colcemid® or colchicine), harvested, stained, and metaphase cells are analysed microscopically for the presence of chromosome aberrations

(f) **Description of the method**—(1) **Preparations**—(i) **Cells**. A variety of cell lines, strains, or primary cell cultures, including human cells, may be used (e.g , Chinese hamster fibroblasts, human, or other mammalian peripheral blood lymphocytes)

(ii) **Media and culture conditions**. Appropriate culture media, and incubation conditions (culture vessels, CO_2 concentration, temperature and humidity) should be used in maintaining cultures. Established cell lines and strains should be checked routinely for stability in the modal chromosome number and the absence of *Mycoplasma* contamination and

should not be used if contaminated The normal cell-cycle time for the cells and culture conditions used should be known

(iii) **Preparation of cultures**—(A) **Established cell lines and strains** Cells are propagated from stock cultures, seeded in culture medium at a density such that the cultures will not reach confluency before the time of harvest, and incubated at 37 °C

(B) **Lymphocytes** Whole blood treated with an anti-coagulant (e g , heparin) or separated lymphocytes obtained from healthy subjects are added to culture medium containing a mitogen (e g , phytohemagglutinin) and incubated at 37 °C

(iv) **Metabolic activation** Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system The most commonly used system is a co-factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 (see paragraphs (i)(6), (i)(7), (8)(i), and (i)(9) of this guideline), or a mixture of phenobarbitone and β-naphthoflavone (see paragraphs (i)(10), (i)(11), and (i)(12) of this guideline). The post-mitochondrial fraction is usually used at concentrations in the range from 1-10 percent v/v in the final test medium The condition of a metabolic activation system may depend upon the class of chemical being tested In some cases, it may be appropriate to utilize more than one concentration of post-mitochondrial fraction A number of developments, including the construction of genetically engineered cell lines expressing specific activating enzymes, may provide the potential for endogenous activation The choice of the cell lines used should be scientifically justified (e g , by the relevance of the cytochrome P450 isoenzyme for the metabolism of the test substance)

(v) **Test substance/preparation** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to treatment of the cells Liquid test substances may be added directly to the test systems and/or diluted prior to treatment Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage

(2) **Test conditions**—(i) **Solvent/vehicle** The solvent/vehicle should not be suspected of chemical reaction with the test substance and should be compatible with the survival of the cells and the S9 activity If other than well-known solvent/vehicles are used, their inclusion should be supported by data indicating their compatibility It is recommended that wherever possible, the use of an aqueous solvent/vehicle be considered first When testing water-unstable substances, the organic solvents used should be free of water Water can be removed by adding a molecular sieve

(ii) **Exposure concentrations** (A) Among the criteria to be considered when determining the highest concentration are cytotoxicity, solubility in the test system and changes in pH or osmolality

(B) Cytotoxicity should be determined with and without metabolic activation in the main experiment using an appropriate indication of cell integrity and growth, such as degree of confluency, viable cell counts, or mitotic index It may be useful to determine cytotoxicity and solubility in a preliminary experiment

(C) At least three analyzable concentrations should be used Where cytotoxicity occurs, these concentrations should cover a range from the maximum to little or no toxicity, this will usually mean that the concentrations should be separated by no more than a factor between 2 and $\sqrt{10}$. At the time of harvesting, the highest concentration should show a significant reduction in degree of confluency, cell count or mitotic index, (all greater than 50 percent) The mitotic index is only an indirect measure of cytotoxic/cytostatic effects and depends on the time after treatment. However, the mitotic index is acceptable for suspension cultures in which other toxicity measurements may be cumbersome and impractical Information on cell-cycle kinetics, such as average generation time (AGT), could be used as supplementary information AGT, however, is an overall average that does not always reveal the existence of delayed subpopulations, and even slight increases in average generation time can be associated with very substantial delay in the time of optimal yield of aberrations. For relatively non-cytotoxic compounds the maximum concentration should be 5 µg/ml, 5mg/ml, or 0 01M, whichever is the lowest.

(D) For relatively insoluble substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used should be a concentration above the limit of solubility in the final culture medium at the end of the treatment period In some cases (e.g , when toxicity occurs only at higher than the lowest insoluble concentration) it is advisable to test at more than one concentration with visible precipitation. It may be useful to assess solubility at the beginning and the end of the treatment, as solubility can change during the course of exposure in the test system due to presence of cells, S9, serum etc Insolubility can be detected by using the unaided eye The precipitate should not interfere with the scoring

(iii) **Controls** (A) Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation should be included in each experiment When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response.

(B) Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over back-

ground which demonstrates the sensitivity of the test system Positive control concentrations should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader Examples of positive-control substances include

Metabolic activation condition	Chemical	CAS number
Absence of exogenous metabolic activation	Methyl methanesulfonate	[66–27–3]
	Ethyl methanesulfonate	[62–50–0]
	Ethylnitrosourea	[759–73–9]
	Mitomycin C	[50–07–7]
	4-Nitroquinoline-N-Oxide	[56–57–5]
Presence of exogenous metabolic activation	Benzo(a)pyrene	[50–32–8]
	Cyclophosphamide (monohydrate)	[50–18–0] ([6055–19–2])

(C) Other appropriate positive control substances may be used The use of chemical class-related positive-control chemicals may be considered, when available

(D) Negative controls, consisting of solvent or vehicle alone in the treatment medium, and treated in the same way as the treatment cultures, should be included for every harvest time In addition, untreated controls should also be used unless there are historical-control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent

(g) **Procedure—(1) Treatment with test substance** (i) Proliferating cells are treated with the test substance in the presence and absence of a metabolic-activation system Treatment of lymphocytes should commence at about 48 hours after mitogenic stimulation

(ii) Duplicate cultures should be used at each concentration, and are strongly recommended for negative/solvent control cultures Where minimal variation between duplicate cultures can be demonstrated (see paragraphs (i)(13) and (i)(14) of this guideline), from historical data, it may be acceptable for single cultures to be used at each concentration.

(iii) Gaseous or volatile substances should be tested by appropriate methods, such as in sealed culture vessels (see paragraphs (i)(15) and (i)(16) of this guideline)

(2) **Culture harvest time** In the first experiment, cells should be exposed to the test substance both with and without metabolic activation for 3–6 hours, and sampled at a time equivalent to about 1.5 normal cell-cycle length after the beginning of treatment (see paragraph (i)(12) of this guideline) If this protocol gives negative results both with and without activation, an additional experiment without activation should be done, with continuous treatment until sampling at a time equivalent to about 1 5 normal cell-cycle lengths Certain chemicals may be more readily detected by treatment/sampling times longer than 1 5 cycle lengths. Negative results with metabolic activation need to be confirmed on a case-by-case

basis In those cases where confirmation of negative results is not considered necessary, justification should be provided

(3) **Chromosome preparation** Cell cultures should be treated with Colcemid® or colchicine usually for 1 to 3 hours prior to harvesting Each cell culture should be harvested and processed separately for the preparation of chromosomes Chromosome preparation involves hypotonic treatment of the cells, fixation and staining

(4) **Analysis** (i) All slides, including those of positive and negative controls, should be independently coded before microscopic analysis Since fixation procedures often result in the breakage of a proportion of metaphase cells with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the modal number ±2 for all cell types At least 200 well-spread metaphases should be scored per concentration and control equally divided amongst the duplicates, if applicable This number can be reduced when high numbers of aberrations are observed.

(ii) Though the purpose of the test is to detect structural chromosome aberrations, it is important to record polyploidy and endoreduplication when these events are seen

(h) **Data and reporting—(1) Treatment of results** (i) The experimental unit is the cell, and therefore the percentage of cells with structural chromosome aberration(s) should be evaluated Different types of structural chromosome aberrations should be listed with their numbers and frequencies for experimental and control cultures Gaps are recorded separately and reported but generally not included in the total aberration frequency

(ii) Concurrent measures of cytotoxicity for all treated and negative control cultures in the main aberration experiment(s) should also be recorded.

(iii) Individual culture data should be provided Additionally, all data should be summarized in tabular form

(iv) There is no requirement for verification of a clear positive response Equivocal results should be clarified by further testing preferably using modification of experimental conditions The need to confirm negative results has been discussed in paragraph (g)(2) of this guideline. Modification of study parameters to extend the range of conditions assessed should be considered in follow-up experiments Study parameters that might be modified include the concentration spacing and the metabolic activation conditions

(2) **Evaluation and interpretation of results** (i) There are several criteria for determining a positive result, such as a concentration-related

increase or a reproducible increase in the number of cells with chromosome aberrations Biological relevance of the results should be considered first Statistical methods may be used as an aid in evaluating the test results (see paragraphs (i)(3) and (i)(13) of this guideline) Statistical significance should not be the only determining factor for a positive response

(ii) An increase in the number of polyploid cells may indicate that the test substance has the potential to inhibit mitotic processes and to induce numerical chromosome aberrations An increase in the number of cells with endoreduplicated chromosomes may indicate that the test substance has the potential to inhibit cell-cycle progression (see paragraphs (i)(17) and (i)(18) of this guideline)

(iii) A test substance for which the results do not meet the criteria in paragraphs (h)(2)(i) and (h)(2)(ii) of this guideline is considered nonmutagenic in this system

(iv) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance Results may remain equivocal or questionable regardless of the number of times the experiment is repeated

(v) Positive results from the *in vitro* chromosome aberration test indicate that the test substance induces structural chromosome aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in cultured mammalian somatic cells

(3) **Test report** The test report should include the following information

(i) Test substance

(A) Identification data and CAS no , if known

(B) Physical nature and purity

(C) Physicochemical properties relevant to the conduct of the study.

(D) Stability of the test substance, if known

(ii) Solvent/vehicle

(A) Justification for choice of solvent/vehicle

(B) Solubility and stability of the test substance in solvent/vehicle, if known

(iii) Cells.

(A) Type and source of cells

(B) Karyotype features and suitability of the cell type used

(C) Absence of *Mycoplasma*, if applicable

(D) Information on cell-cycle length

(E) Sex of blood donors, whole blood or separated lymphocytes, mitogen used

(F) Number of passages, if applicable

(G) Methods for maintenance of cell cultures if applicable.

(H) Modal number of chromosomes

(iv) Test conditions

(A) Identity of metaphase arresting substance, its concentration and duration of cell exposure

(B) Rationale for selection of concentrations and number of cultures including, e g , cytotoxicity data and solubility limitations, if available

(C) Composition of media, CO_2 concentration if applicable.

(D) Concentration of test substance

(E) Volume of vehicle and test substance added

(F) Incubation temperature

(G) Incubation time

(H) Duration of treatment

(I) Cell density at seeding, if appropriate

(J) Type and composition of metabolic activation system, including acceptability criteria.

(K) Positive and negative controls

(L) Methods of slide preparation

(M) Criteria for scoring aberrations

(N) Number of metaphases analyzed

(O) Methods for the measurements of toxicity

(P) Criteria for considering studies as positive, negative or equivocal

(v) Results

(A) Signs of toxicity e g , degree of confluency cell-cycle data cell counts, mitotic index

(B) Signs of precipitation

(C) Data on pH and osmolality of the treatment medium, if determined

(D) Definition for aberrations, including gaps

(E) Number of cells with chromosome aberrations and type of chromosome aberrations given separately for each treated and control culture

(F) Changes in ploidy if seen

(G) Dose-response relationship, where possible

(H) Statistical analyses, if any

(I) Concurrent negative (solvent/vehicle) and positive control data

(J) Historical negative (solvent/vehicle) and positive control data, with ranges, means and standard deviations

(vi) Discussion of the results

(vii) Conclusion.

(i) **References.** The following references should be consulted for additional background information on this test guideline

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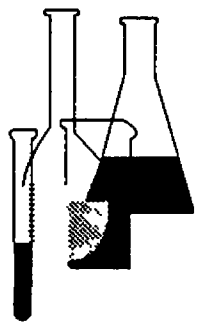
Health Effects Test Guidelines

OPPTS 870.5380 Mammalian Spermatogonial Chromosome Aberration Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S C 136, *et seq*)

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OPPTS 870 5380 Mammalian spermatogonial chromosome aberration test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline is OECD 483, Mammalian Spermatogonial Chromosome Aberration Test

(b) **Purpose** (1) The purpose of the *in vivo* mammalian spermatogonial chromosome aberration test is to identify those substances that cause structural aberrations in mammalian spermatogonial cells (see paragraphs (i)(1), (i)(2), (i)(3), (i)(4), and (i)(5) of this guideline). Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur This guideline is not designed to measure numerical aberrations and is not routinely used for this purpose Chromosome mutations and related events are the cause of many human genetic diseases

(2) This test measures chromosome events in spermatogonial germ cells and is, therefore, expected to be predictive of induction of inheritable mutations in germ cells

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Chromatid-type aberration is structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration is structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site

Gap is an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids

Numerical aberration is a change in the number of chromosomes from the normal number characteristic of the animals utilized

Polyploidy is a multiple of the haploid chromosome number (n) other than the diploid number (i e , 3n, 4n, and so on)

Structural aberration is a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions, intrachanges or interchanges

(d) **Initial considerations** (1) Rodents are routinely used in this test This *in vivo* cytogenetic test detects chromosome aberrations in spermatogonial mitoses Other target cells are not the subject of this guideline

(2) To detect chromatid-type aberrations in spermatogonial cells, the first mitotic cell division following treatment should be examined before these lesions are lost in subsequent cell divisions Additional information from treated spermatogonial stem cells can be obtained by meiotic chromosome analysis for chromosome-type aberrations at diakinesis-metaphase I when the treated cells become spermatocytes.

(3) This *in vivo* test is designed to investigate whether somatic cell mutagens are also active in germ cells In addition, the spermatogonial test is relevant to assessing mutagenicity hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes

(4) A number of generations of spermatogonia are present in the testis with a spectrum of sensitivity to chemical treatment Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations, with the more numerous differentiated spermatogonial cells predominating Depending on their position within the testis, different generations of spermatogonia may or may not be exposed to the general circulation, because of the physical and physiological Sertoli cell barrier and the blood-testis barrier

(5) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test

(e) **Principle of the test method** Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment Prior to sacrifice, animals are treated with a metaphase-arresting agent (e g , colchicine or Colcemid®). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analyzed for chromosome aberrations

(f) **Description of the method—(1) Preparations—(i) Selection of animal species** Male Chinese hamsters and mice are commonly used However, males of other appropriate mammalian species may be used. Commonly used laboratory strains of healthy young-adult animals should be employed At the commencement of the study the weight variation of animals should be minimal and not exceed ±20 percent of the mean weight

(ii) **Housing and feeding conditions.** The temperature in the experimental animal room should be 22 °C (±3 °C) Although the relative humidity should be at least 30 percent and preferably not exceed 70 percent

other than during room cleaning, the aim should be 50–60 percent Lighting should be artificial, the sequence being 12 hours light, 12 hours dark For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method Animals may be housed individually, or be caged in small groups

(iii) **Preparation of the animals** Healthy young-adult males should be randomly assigned to the control and treatment groups Cages should be arranged in such a way that possible effects due to cage placement are minimized The animals are identified uniquely The animals are acclimated to the laboratory conditions for at least 5 days prior to the start of the study

(iv) **Preparation of doses** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals Liquid test substances may be dosed directly or diluted prior to dosing Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage

(2) **Test conditions—(i) Solvent/vehicle** The solvent/vehicle should not produce toxic effects at the dose levels used and should not be suspected of chemical reaction with the test substance If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(ii) **Controls** (A) Concurrent positive and negative (solvent/vehicle) controls should be included in each test Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals in the treated groups

(B) Positive controls should produce structural chromosome aberrations *in vivo* in spermatogonial cells when administered at exposure levels expected to give a detectable increase over background Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time In addition, the use of chemical class-related positive control chemicals may be considered, when available Examples of positive control substances include.

Chemical	CAS number
Cyclophosphamide (monohydrate)	[50-18-0 (6055-19-2)]
Cyclohexylamine	[108-91-8]
Mitomycin C	[50-07-7]
Monomeric acrylamide	[79-06-1]
Triethylenemelamine	[51-18-3]

(C) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequency of cells with chromosome aberrations are demonstrated by historical control data In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle.

(g) **Procedure**—(1) **Number of animals** Each treated and control group should include at least five analyzable males

(2) **Treatment schedule** (i) Test substances are preferably administered once or twice (i e as a single treatment or as two treatments) Test substances may also be administered as a split dose, i e two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material Other dose regimens should be scientifically justified

(ii) In the highest dose group, two sampling times after treatment should be used Since cell cycle kinetics can be influenced by the test substance, one early and one late sampling time are used around 24 and 48 hours after treatment For doses other than the highest dose, a sampling time of 24 hours or 1 5 cell cycle length after treatment should be taken, unless another sampling time is known to be more appropriate for detection of effects (see paragraph (i)(6) of this guideline)

(iii) In addition, other sampling times may be used For example, in the case of chemicals which may induce chromosome lagging, or may exert S-independent effects, earlier sampling times may be appropriate (see paragraph (i)(1) of this guideline)

(iv) The appropriateness of a repeated treatment schedule needs to be identified on a case-by-case basis Following a repeated treatment schedule the animals should then be sacrificed 24 hours (1.5 cell-cycle length) after the last treatment Additional sampling times may be used where appropriate

(v) Prior to sacrifice, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting substance (e g , Colcemid® or colchicine) Animals are sampled at an appropriate interval thereafter For

mice this interval is approximately 3–5 hours, for Chinese hamsters this interval is approximately 4–5 hours

(3) **Dose levels** If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, and treatment regimen to be used in the main study (see paragraph (i)(7) of this guideline) If there is toxicity, three-dose levels are used for the first sampling time These dose levels should cover a range from the maximum to little or no toxicity At the later sampling time only the highest dose needs to be used The highest dose is defined as the dose producing signs of toxicity such that higher-dose levels, based on the same dosing regimen, would be expected to produce lethality Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis The highest dose may also be defined as a dose that produces some indication of toxicity in the spermatogonial cells (e g , a reduction in the ratio of spermatogonial mitoses to first and second meiotic metaphases, this reduction should not exceed 50 percent)

(4) **Limit test** If a test at one dose level of at least 2,000 mg/kg body weight/day using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three-dose levels may not be considered necessary Expected human exposure may indicate the need for a higher dose level to be used in the limit test

(5) **Administration of doses** The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection Other routes of exposure may be acceptable where they can be justified The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified Except for irritating or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels

(6) **Chromosome preparation** Immediately after sacrifice, cell suspensions should be obtained from one or both testes, exposed to hypotonic solution and fixed The cells should be then spread on slides and stained.

(7) **Analysis** For each animal at least 100 well-spread metaphases should be analyzed (i e a minimum of 500 metaphases per group) This number could be reduced when high numbers of aberrations are observed All slides, including those of positive and negative controls, should be

independently coded before microscopic analysis Since fixation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should contain a number of centromeres equal to the number $2n \pm 2$

(h) **Data and reporting**—(1) **Treatment of results** (i) Individual animal data should be presented in tabular form The experimental unit is the animal For each animal the number of cells with structural chromosome aberration(s) and the number of chromosome aberrations per cell should be evaluated Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups. Gaps are recorded separately and reported but generally not included in the total aberration frequency

(ii) If mitosis as well as meiosis is observed, the ratio of spermatogonial mitoses to first and second meiotic metaphases should be determined as a measure of cytotoxicity for all treated and negative control animals in a total sample of 100 dividing cells per animal to establish a possible cytotoxic effect If only mitosis is observed, the mitosis index should be determined in at least 1,000 cells for each animal

(2) **Evaluation and interpretation of results** (i) There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single-dose group at a single-sampling time Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results (see paragraph (i)(8) of this guideline). Statistical significance should not be the only determining factor for a positive response Equivocal results should be clarified by further testing preferably using a modification of experimental conditions

(ii) A test substance for which the results do not meet the criteria in paragraph (h)(2)(i) of this guideline is considered nonmutagenic in this test

(iii) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance Results may remain equivocal or questionable regardless of the number of times the experiment is repeated.

(iv) Positive results from the *in vivo* spermatogonial chromosome aberration test indicate that a substance induces chromosome aberrations in the germ cells of the species tested Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the germ cells of the species tested

(v) The likelihood that the test substance or its metabolites reach the target tissue should be discussed

(3) **Test report** The test report should include the following information

(i) Test substance

(A) Identification data and CAS No , if known

(B) Physical nature and purity

(C) Physicochemical properties relevant to the conduct of the study

(D) Stability of the test substance, if known

(ii) Solvent/vehicle

(A) Justification for choice of vehicle

(B) Solubility and stability of the test substance in solvent/vehicle, if known

(iii) Test animals

(A) Species/strain used

(B) Number and age of animals

(C) Source, housing conditions, diet, etc

(D) Individual weight of the animals at the start of the test, including body weight range, mean, and standard deviation for each group.

(iv) Test conditions

(A) Data from range finding study, if conducted

(B) Rationale for dose level selection

(C) Rationale for route of administration

(D) Details of test substance preparation

(E) Details of the administration of the test substance

(F) Rationale for sacrifice times

(G) Conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable

(H) Details of food and water quality

(I) Detailed description of treatment and sampling schedules

(J) Methods for measurement of toxicity

(K) Identity of metaphase arresting substance its concentration and duration of treatment

(L) Methods of slide preparation

(M) Criteria for scoring aberrations

(N) Number of cells analyzed per animal

(O) Criteria for considering studies as positive, negative, or equivocal

(v) Results

(A) Signs of toxicity

(B) Mitotic index

(C) Ratio of spermatogonial mitoses cells to first and second meiotic metaphases

(D) Type and number of aberrations, given separately for each animal

(E) Total number of aberrations per group

(F) Number of cells with aberrations per group

(G) Dose-response relationship, where possible

(H) Statistical analyses, if any.

(I) Concurrent negative control data

(J) Historical negative control data with ranges, means, and standard deviations

(K) Concurrent positive control data

(L) Changes in ploidy, if seen.

(vi) Discussion of the results

(vii) Conclusion.

(i) **References.** The following references should be consulted for additional background information on this test guideline

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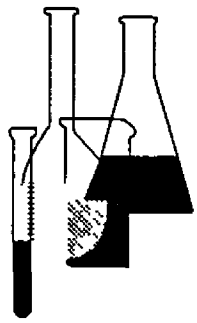
Health Effects Test Guidelines OPPTS 870.5385 Mammalian Bone Marrow Chromosome Aberration Test



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OPPTS 870.5385 Mammalian bone marrow chromosome aberration test

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5385 In vivo mammalian cytogenetics test Bone marrow chromosomal analysis and OECD 475, Mammalian Bone Marrow Chromosome Aberration Test

(b) **Purpose.** The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of animals, usually rodents (see references in paragraphs (g)(1), (g)(2), (g)(3), and (g)(4) of this guideline) Structural chromosome aberrations may be of two types, chromosome or chromatid An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur Chromosome mutations and related events are the cause of many human-genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes are involved in cancer in humans and experimental systems

(c) **Definitions.** The following definitions apply to the guideline

Chromatid-type aberration is structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids

Chromosome-type aberration is structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Endoreduplication is a process in which after an S period of DNA replication, the nucleus does not go into mitosis but starts another S period The result is chromosomes with 2,4,8, . chromatids.

Gap is an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Numerical aberration is a change in the number of chromosomes from the normal number characteristic of the animals utilized

Polyploidy is a multiple of the haploid chromosome number (n) other than the diploid number (i e , 3n, 4n, and so on)

Structural aberration is a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges or interchanges

(d) **Initial considerations.** (1) Rodents are routinely used in this test Bone marrow is the target tissue in this test, since it is a highly vascularized tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed Other species and target tissues are not the subject of this guideline

(2) This chromosome aberration test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes although these may vary among species and among tissues An *in vivo* test is also useful for further investigation of a mutagenic effect detected by an *in vitro* test

(3) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test

(e) **Test method**—(1) **Principle.** Animals are exposed to the test substance by an appropriate route of exposure and are sacrificed at appropriate times after treatment. Prior to sacrifice, animals are treated with a metaphase-arresting agent (e g , colchicine or Colcemid®) Chromosome preparations are then made from the bone marrow cells and stained, and metaphase cells are analyzed for chromosome aberrations

(2) **Description**—(i) **Preparations**—(A) **Selection of animal species.** Rats, mice, and Chinese hamsters are commonly used, although any appropriate mammalian species may be used Commonly used laboratory strains of healthy young-adult animals should be employed At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20 percent of the mean weight of each sex

(B) **Housing and feeding conditions.** The temperature in the experimental animal room should be 22 °C (±3 °C) Although the relative humidity should be at least 30 percent and preferably not exceed 70 percent other than during room cleaning, the aim should be 50–60 percent Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method Animals may be housed individually, or be caged in small groups of the same sex

(C) **Preparation of the animals.** Healthy, young-adult animals should be randomly assigned to the control and treatment groups Cages should be arranged in such a way that possible effects due to cage placement

are minimized The animals are identified uniquely The animals are acclimated to the laboratory conditions for at least 5 days

(D) **Preparation of doses.** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals Liquid-test substances may be dosed directly or diluted prior to dosing Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage

(ii) **Test conditions**—(A) **Solvent/vehicle.** The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance If other than well-known solvents/vehicles are used, their inclusion should be supported with data indicating their compatibility It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(B) **Controls.** (*1*) Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to the animals in the treated groups

(*2*) Positive controls should produce structural chromosome aberrations *in vivo* at exposure levels expected to give a detectable increase over background Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time The use of chemical class related positive control chemicals may be considered, when available Examples of positive control substances include.

Chemical	CAS number
Triethylenemelamine	[51–18–3]
Ethyl methanesulphonate	[62–50–0]
Ethyl nitrosourea	[759–73–9]
Mitomycin C	[50–07–7]
Cyclophosphamide (monohydrate)	[50–18–0] ([6055–19–2])

(*3*) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with chromosome aberrations are available from historical control data. If single sampling is applied for negative controls, the most appropriate time is the first sampling time In addition, untreated controls should also be used unless there are historical or published control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle

(3) **Procedure**—(i) **Number and sex of animals.** Each treated and control group should include at least five analyzable animals per sex If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient Where human exposure to chemicals may be sex specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex

(ii) **Treatment schedule.** (A) Test substances are preferably administered as a single treatment Test substances may also be administered as a split dose, i.e two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material Other dose regimens should be scientifically justified

(B) Samples should be taken at two separate times following treatment on one day For rodents, the first sampling interval is 1 5 normal cell-cycle length (the latter being normally 12–18 hours) following treatment Since the time required for uptake and metabolism of the test substance as well as its effect on cell-cycle kinetics can affect the optimum time for chromosome aberration detection, a later sample collection 24 hours after the first sample time is recommended If dose regimens of more than 1 day are used, one sampling time at 1 5 normal cell-cycle lengths after the final treatment should be used

(C) Prior to sacrifice, animals should be injected intraperitoneally with an appropriate dose of a metaphase arresting agent (e g., Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3–5 hours, for Chinese hamsters this interval is approximately 4–5 hours Cells should be harvested from the bone marrow and analysed from chromosome aberrations.

(iii) **Dose levels.** If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (see reference in paragraph (g)(5) of this guideline) If there is toxicity, three-dose levels should be used for the first sampling time. These dose levels should cover a range from the maximum to little or no toxicity At the later sampling time only the highest dose needs to be used. The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e.g , greater than 50 percent reduction in mitotic index)

(iv) **Limit test.** If a test at one dose level of at least 2,000 mg/kg body weight using a single treatment or as two treatments on the same day produces no observable toxic effects, and if genotoxicity would not be expected based on data from structurally related compounds then a full study using three-dose levels may not be considered necessary For studies of a longer duration, the limit dose is 2,000 mg/kg/body weight/ day for treatment up to 14 days, and 1,000 mg/kg/body weight/day for treatment longer than 14 days Expected human exposure may indicate the need for a higher-dose level to be used in the limit test

(v) **Administration of doses.** The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection Other routes of exposure may be acceptable where they can be justified The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal The volume should not exceed 2 ml/100g body weight The use of volumes higher than these must be justified Except for irritating or corrosive substances which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels.

(vi) **Chromosome preparation.** Immediately after sacrifice, bone marrow should be obtained, exposed to hypotonic solution and fixed The cells should be then spread on slides and stained

(vii) **Analysis.** (A) The mitotic index should be determined as a measure of cytotoxicity in at least 1,000 cells per animal for all treated animals (including positive controls) and untreated negative control animals

(B) At least 100 cells should be analyzed for each animal. This number could be reduced when high numbers of aberrations are observed All slides, including those of positive and negative controls, should be independently coded before microscopic analysis Since slide preparation procedures often result in the breakage of a proportion of metaphases with loss of chromosomes, the cells scored should therefore contain a number of centromeres equal to the number 2n ±2.

(f) **Data and reporting—(1) Treatment of results.** Individual animal data should be presented in tabular form The experimental unit is the animal For each animal the number of cells scored, the number of aberrations per cell and the percentage of cells with structural chromosome aberration(s) should be evaluated Different types of structural chromosome aberrations should be listed with their numbers and frequencies for treated and control groups Gaps should be recorded separately and reported but generally not included in the total aberration frequency If there is no evidence for a difference in response between the sexes, the data may be combined for statistical analysis

(2) **Evaluation and interpretation of results.** (i) There are several criteria for determining a positive result, such as a dose-related increase in the relative number of cells with chromosome aberrations or a clear increase in the number of cells with aberrations in a single-dose group at a single-sampling time Biological relevance of the results should be considered first Statistical methods may be used as an aid in evaluating the test results (see reference in paragraph (g)(6) of this guideline) Statistical significance should not be the only determining factor for a positive response Equivocal results should be clarified by further testing preferably using a modification of experimental conditions

(ii) An increase in polyploidy may indicate that the test substance has the potential to induce numerical chromosome aberrations An increase in endoreduplication may indicate that the test substance has the potential to inhibit cell-cycle progression (see references in paragraphs (g)(7) and (g)(8) of this guideline)

(iii) A test substance for which the results do not meet the criteria in paragraphs (f)(2)(i) and (f)(2)(ii) of this guideline is considered nonmutagenic in this test

(iv) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance Results may remain equivocal or questionable regardless of the number of experiments performed

(v) Positive results from the *in vivo* chromosome aberration test indicate that a substance induces chromosome aberrations in the bone marrow of the species tested Negative results indicate that, under the test conditions, the test substance does not induce chromosome aberrations in the bone marrow of the species tested

(vi) The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e g , systemic toxicity) should be discussed

(3) **Test report.** The test report should include the following information

(i) Test substance·

(A) Identification data and CAS No , if known

(B) Physical nature and purity

(C) Physicochemical properties relevant to the conduct of the study

(D) Stability of the test substance, if known

(ii) Solvent/vehicle

(A) Justification for choice of vehicle

(B) Solubility and stability of the test substance in solvent/vehicle, if known

(iii) Test animals

(A) Species/strain used

(B) Number, age, and sex of animals

(C) Source, housing conditions, diet, etc

(D) Individual weight of the animals at the start of the test, including body weight range, mean, and standard deviation for each group

(iv) Test conditions

(A) Positive and negative (vehicle/solvent) controls

(B) Data from range-finding study, if conducted

(C) Rationale for dose level selection

(D) Details of test substance preparation

(E) Details of the administration of the test substance

(F) Rationale for route of administration

(G) Methods for verifying that the test substance reached the general circulation or target tissue, if applicable

(H) Conversion from diet/drinking water test substance concentration parts per million (ppm) to the actual dose (mg/kg body weight/day), if applicable

(I) Details of food and water quality

(J) Detailed description of treatment and sampling schedules.

(K) Methods for measurement of toxicity

(L) Identity of metaphase arresting substance, its concentration and duration of treatment

(M) Methods of slide preparation

(N) Criteria for scoring aberrations

(O) Number of cells analysed per animal

(P) Criteria for considering studies as positive, negative or equivocal

(v) Results

(A) Signs of toxicity

(B) Mitotic index

(C) Type and number of aberrations, given separately for each animal

(D) Total number of aberrations per group with means and standard deviations

(E) Number of cells with aberrations per group with means and standard deviations

(F) Changes in ploidy, if seen

(G) Dose-response relationship, where possible

(H) Statistical analyses, if any

(I) Concurrent negative control data

(J) Historical negative control data with ranges, means and standard deviations.

(K) Concurrent positive control data

(vi) Discussion of the results

(vii) Conclusion

(g) **References.** The following references should be consulted for additional background information on this test guideline

(1) Adler, I.D Cytogenetic Tests in Mammals Mutagenicity Testing: A Practical Approach (Eds) S Venitt and J M Parry (IRL Press, Oxford, Washington DC, pp 275–306 (1984)

(2) Preston, R.J et al Mammalian *In Vivo* Cytogenetic Assays: Analysis of Chromosome Aberrations in Bone Marrow Cells *Mutation Research* 189, 157–165 (1987)

(3) Richold, M. et al *In Vivo* Cytogenetic Assays (Ed) D J. Kirkland Basic Mutagenicity Tests, UKEMS Recommended Procedures. UKEMS Subcommittee on Guidelines for Mutagenicity Testing Report. Part I revised (Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp 115–141 (1990)

(4) Tice, R.R et al Report from the Working Group on the *In Vivo* Mammalian Bone Marrow Chromosomal Aberration Test *Mutation Research* 312, 305–312 (1994)

(5) Fielder, R J et al Report of British Toxicology Society/UK Environmental Mutagen Society Working Group Dose Setting in *In Vivo* Mutagenicity Assays *Mutagenesis* 7, 313–319 (1992)

(6) Lovell, D P et al Statistical Analysis of *In Vivo* Cytogenetic Assays UKEMS Sub-Committee on Guidelines for Mutagenicity Testing Report Part III Statistical Evaluation of Mutagenicity Test Data (Ed) D J Kirkland (Cambridge University Press, Cambridge, pp 184–232 (1989)

(7) Locke-Huhle, C Endoreduplication in Chinese Hamster Cells During Alpha-Radiation Induced G2 Arrest *Mutation Research* 119, 403–413 (1983)

(8) Huang, Y et al Aphidicolin-Induced Endoreduplication in Chinese Hamster Cells *Cancer Research* 43, 1362–1364 (1983)

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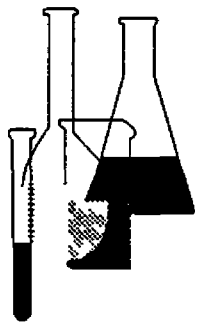
Health Effects Test Guidelines OPPTS 870.5395 Mammalian Erythrocyte Micronucleus Test



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S Environmental Protection Agency under the Toxic Substances Control Act (15 U.S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870 5395 Mammalian erythrocyte micronucleus test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5385 In vivo mammalian bone marrow cytogenetics test Micronucleus assay and OECD 474, Mammalian Erythrocyte Micronucleus Test

(b) **Purpose.** (1) The mammalian *in vivo* micronucleus test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals, usually rodents

(2) The purpose of the micronucleus test is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

(3) When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded, any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualization of micronuclei is facilitated in these cells because they lack a main nucleus An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage

(c) **Definitions** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Centromere (kinetochore) is a region of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells

Micronuclei are small nuclei, separate from and additional to the main nuclei of cells, produced during telophase of mitosis (meiosis) by lagging chromosome fragments or whole chromosomes

Normochromatic erythrocyte is a mature erythrocyte that lacks ribosomes and can be distinguished from immature, polychromatic erythrocytes by stains selective for ribosomes

Polychromatic erythrocyte is a immature erythrocyte, in an intermediate stage of development, that still contains ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes by stains selective for ribosomes

(d) **Initial considerations.** (1) The bone marrow of rodents is routinely used in this test since polychromatic erythrocytes are produced in that tissue The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations Micronuclei can be distinguished by a number of criteria These include identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei The frequency of micronucleated immature (polychromatic) erythrocytes is the principal endpoint The number of mature (normochromatic) erythrocytes in the peripheral blood that contain micronuclei among a given number of mature erythrocytes can also be used as the endpoint of the assay when animals are treated continuously for 4 weeks or more This mammalian *in vivo* micronucleus test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes although these may vary among species, among tissues and among genetic endpoints An *in vivo* assay is also useful for further investigation of a mutagenic effect detected by an *in vitro* system

(2) If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test

(e) **Test method**—(1) **Principle.** Animals are exposed to the test substance by an appropriate route If bone marrow is used, the animals are sacrificed at appropriate times after treatment, the bone marrow extracted, and preparations made and stained (see references in paragraphs (g)(1), (g)(2), and (g)(3) of this guideline) When peripheral blood is used, the blood is collected at appropriate times after treatment and smear preparations are made and stained (see references in paragraphs (g)(3), (g)(4), (g)(5), and (g)(6) of this guideline) For studies with peripheral blood, as little time as possible should elapse between the last exposure and cell harvest. Preparations are analyzed for the presence of micronuclei.

(2) **Description**—(i) **Preparations**—(A) **Selection of animal species.** Mice or rats are recommended if bone marrow is used, although any appropriate mammalian species may be used When peripheral blood is used, mice are recommended However, any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes or a species which has shown an adequate sensitivity to detect agents that cause structural or numerical chromosome aberrations Commonly used laboratory strains of healthy young animals should be employed At the commencement of the study, the weight variation of animals should be minimal and not exceed ±20 percent of the mean weight of each sex

(B) **Housing and feeding conditions.** The temperature in the experimental animal room should be 22 °C (±3 °C) Although the relative humidity should be at least 30 percent and preferably not exceed 70 percent other than during room cleaning, the aim should be 50–60 percent Lighting should be artificial, the sequence being 12 hours light, 12 hours dark For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this route Animals may be housed individually, or caged in small groups of the same sex

(C) **Preparation of the animals.** Healthy young adult animals should be randomly assigned to the control and treatment groups The animals are identified uniquely The animals are acclimated to the laboratory conditions for at least five days Cages should be arranged in such a way that possible effects due to cage placement are minimized

(D) **Preparation of doses.** Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals Liquid-test substances may be dosed directly or diluted prior to dosing Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

(ii) **Test conditions**—(A) **Solvent/vehicle.** The solvent/vehicle should not produce toxic effects at the dose levels used, and should not be suspected of chemical reaction with the test substance If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first.

(B) **Controls.** (*1*) Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each test Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups

(2) Positive controls should produce micronuclei *in vivo* at exposure levels expected to give a detectable increase over background. Positive-control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. It is acceptable that the positive control be administered by a route different from the test substance and sampled at only a single time In addition, the use of chemical class-related positive control chemicals may be considered, when available Examples of positive control substances include.

Chemical	CAS number
Ethyl methanesulphonate	[62-50-0]
Ethyl nitrosourea	[759-73-9]
Mitomycin C	[50-07-7]
Cyclophosphamide (monohydrate)	[50-18-0] ([6055-19-2])
Triethylenemelamine	[51-18-3]

(*3*) Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups should be included for every sampling time, unless acceptable inter-animal variability and frequencies of cells with micronuclei are demonstrated by historical-control data If single sampling is applied for negative controls, the most appropriate time is the first sampling time In addition, untreated controls should also be used unless there are historical- or published-control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent/vehicle

(*4*) If peripheral blood is used, a pre-treatment sample may also be acceptable as a concurrent negative control, but only in the short peripheral blood studies (e g , one–three treatment(s)) when the resulting data are in the expected range for the historical control

(3) **Procedure**—(i) **Number and sex of animals.** Each treated and control group should include at least 5 analyzable animals per sex (see reference in paragraph (g)(7) of this guideline) If at the time of the study there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences between sexes in toxicity, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex

(ii) **Treatment schedule.** (A) No standard treatment schedule (i.e 1, 2, or more treatments at 24 hour intervals) can be recommended. The samples from extended dose regimens are acceptable as long as a positive effect has been demonstrated for this study or, for a negative study, as long as toxicity has been demonstrated or the limit dose has been used, and dosing continued until the time of sampling Test substances may also be administered as a split dose, i e , two treatments on the same day separated by no more than a few hours, to facilitate administering a large volume of material

(B) The test may be performed in two ways.

(*1*) Animals should be treated with the test substance once. Samples of bone marrow should be taken at least twice, starting not earlier than 24 hour after treatment, but not extending beyond 48 hours after treatment with appropriate interval(s) between samples The use of sampling times earlier than 24 hours after treatment should be justified Samples of periph-

eral blood should be taken at least twice, starting not earlier than 36 hours after treatment, with appropriate intervals following the first sample, but not extending beyond 72 hours When a positive response is recognized at one sampling time, additional sampling is not required

(2) If two or more daily treatments are used (e g two or more treatments at 24 hour intervals), samples should be collected once between 18 and 24 hours following the final treatment for the bone marrow and once between 36 and 48 hours following the final treatment for the peripheral blood (see reference in paragraph (g)(8) of this guideline)

(C) Other sampling times may be used in addition, when relevant

(iii) **Dose levels.** If a range finding study is performed because there are no suitable data available, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (see reference in paragraph (g)(9) of this guideline) If there is toxicity, three-dose levels should be used for the first sampling time These dose levels should cover a range from the maximum to little or no toxicity At the later sampling time only the highest dose needs to be used The highest dose is defined as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis The highest dose may also be defined as a dose that produces some indication of toxicity in the bone marrow (e g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood).

(iv) **Limit test.** If a test at one dose level of at least 2,000 mg/kg body weight using a single treatment, or as two treatments on the same day, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three-dose levels may not be considered necessary. For studies of a longer duration, the limit dose is 2,000 mg/kg/body weight/ day for treatment up to 14 days, and 1,000 mg/kg/body weight/day for treatment longer than 14 days Expected human exposure may indicate the need for a higher-dose level to be used in the limit test

(v) **Administration of doses.** The test substance is usually administered by gavage using a stomach tube or a suitable intubation cannula, or by intraperitoneal injection Other routes of exposure may be acceptable where they can be justified The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal The volume should not exceed 2 ml/100g body weight. The use of volumes higher than these must be justified. Except for irritating or corrosive substances which will normally reveal exacerbated effects

with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels

(vi) **Bone marrow/blood preparation.** Bone marrow cells should be obtained from the femurs or tibias immediately following sacrifice Cells should be removed from femurs or tibias, prepared and stained using established methods Peripheral blood is obtained from the tail vein or other appropriate blood vessel Blood cells are immediately stained supravitally (see references in paragraphs (g)(4), (g)(5), and (g)(6) of this guideline) or smear preparations are made and then stained The use of a DNA-specific stain (e g acridine orange (see reference in paragraph (g)(10) of this guideline) or Hoechst 33258 plus pyronin-Y (see reference in paragraph (g)(11) of this guideline) can eliminate some of the artifacts associated with using a non-DNA-specific stain This advantage does not preclude the use of conventional stains (e g , Giemsa) Additional systems (e g cellulose columns to remove nucleated cells (see reference in paragraph (g)(12) of this guideline) can also be used provided that these systems have been shown to adequately work for micronucleus preparation in the laboratory

(vii) **Analysis.** The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1,000 erythrocytes for peripheral blood (see reference in paragraph (g)(13) of this guideline) All slides, including those of positive and negative controls, should be independently coded before microscopic analysis. At least 2,000 immature erythrocytes per animal should be scored for the incidence of micronucleated immature erythrocytes Additional information may be obtained by scoring mature erythrocytes for micronuclei. When analyzing slides, the proportion of immature erythrocytes among total erythrocytes should not be less than 20 percent of the control value. When animals are treated continuously for 4 weeks or more, at least 2,000 mature erythrocytes per animal can also be scored for the incidence of micronuclei Systems for automated analysis (image analysis) and cell suspensions (flow cytometry) are acceptable alternatives to manual evaluation if appropriately justified and validated

(f) **Data and reporting—(1) Treatment of results.** Individual animal data should be presented in tabular form. The experimental unit is the animal The number of immature erythrocytes scored, the number of micronucleated immature erythrocytes, and the number of immature among total erythrocytes should be listed separately for each animal analyzed. When animals are treated continuously for 4 weeks or more, the data on mature erythrocytes should also be given if it is collected. The proportion of immature among total erythrocytes and, if considered applicable, the percentage of micronucleated erythrocytes should be given for each animal If there is no evidence for a difference in response between

the sexes, the data from both sexes may be combined for statistical analysis

(2) **Evaluation and interpretation of results.** (i) There are several criteria for determining a positive result, such as a dose-related increase in the number of micronucleated cells or a clear increase in the number of micronucleated cells in a single-dose group at a single-sampling time Biological relevance of the results should be considered first Statistical methods may be used as an aid in evaluating the test results (see references in paragraphs (g)(14) and (g)(15) of this guideline) Statistical significance should not be the only determining factor for a positive response. Equivocal results should be clarified by further testing preferably using a modification of experimental conditions

(ii) A test substance for which the results do not meet the criteria in paragraph (f)(2)(i) of this guideline is considered non-mutagenic in this test

(iii) Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance Results may remain equivocal or questionable regardless of the number of times the experiment is repeated Positive results in the micronucleus test indicate that a substance induces micronuclei which are the result of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. Negative results indicate that, under the test conditions, the test substance does not produce micronuclei in the immature erythrocytes of the test species

(iv) The likelihood that the test substance or its metabolites reach the general circulation or specifically the target tissue (e g systemic toxicity) should be discussed

(3) **Test report.** The test report should include the following information·

(i) Test substance

(A) Identification data and CAS No , if known

(B) Physical nature and purity

(C) Physiochemical properties relevant to the conduct of the study

(D) Stability of the test substance, if known

(ii) Solvent/vehicle

(A) Justification for choice of vehicle

(B) Solubility and stability of the test substance in the solvent/vehicle, if known

(iii) Test animals

(A) Species/strain used

(B) Number, age, and sex of animals

(C) Source, housing conditions, diet, etc

(D) Individual weight of the animals at the start of the test, including body weight range, mean, and standard deviation for each group

(iv) Test conditions

(A) Positive and negative (vehicle/solvent) control data

(B) Data from range-finding study, if conducted

(C) Rationale for dose-level selection

(D) Details of test substance preparation

(E) Details of the administration of the test substance

(F) Rationale for route of administration

(G) Methods for verifying that the test substance reached the general circulation or target tissue, if applicable

(H) Conversion from diet/drinking water test substance concentration parts per million (ppm) to the actual dose (mg/kg body weight/day), if applicable

(I) Details of food and water quality

(J) Detailed description of treatment and sampling schedules

(K) Methods of slide preparation

(L) Methods for measurement of toxicity

(M) Criteria for scoring micronucleated immature erythrocytes

(N) Number of cells analyzed per animal.

(O) Criteria for considering studies as positive, negative, or equivocal.

(v) Results

(A) Signs of toxicity.

(B) Proportion of immature erythrocytes among total erythrocytes

(C) Number of micronucleated immature erythrocytes, given separately for each animal

(D) Mean ± standard deviation of micronucleated immature erythrocytes per group

(E) Dose-response relationship, where possible

(F) Statistical analyses and method applied

(G) Concurrent and historical negative-control data

(H) Concurrent positive-control data

(vi) Discussion of the results

(vii) Conclusion

(g) **References.** The following references should be consulted for additional background information on this test guideline

(1) Heddle, J A et al A Rapid *In vivo* Test for Chromosomal Damage *Mutation Research* 18, 187–190 (1973)

(2) Schmid, W The Micronucleus Test *Mutation Research* 31, 9–15 (1975)

(3) Mavournin, K H et al The *In vivo* Micronucleus Assay in Mammalian Bone Marrow and Peripheral Blood A report of the U S. Environmental Protection Agency Gene-Tox Program *Mutation Research* 239, 29–80 (1990)

(4) Hayashi, M et al The Micronucleus Assay with Mouse Peripheral Blood Reticulocytes Using Acridine Orange-Coated Slides *Mutation Research* 245, 245–249 (1990)

(5) The Collaborative Study Group for the Micronucleus Test Micronucleus Test with Mouse Peripheral Blood Erythrocytes by Acridine Orange Supravital Staining The Summary Report of the 5th Collaborative Study by CSGMT/JEMS MMS *Mutation Research* 278, 83–98 (1992).

(6) The Collaborative Study Group for the Micronucleus Test (CSGMT/JEMMS MMS, The Mammalian Mutagenesis Study Group of the Environmental Mutagen Society of Japan) Protocol recommended for the short-term mouse peripheral blood micronucleus test *Mutagenesis* 10, 153–159 (1995)

(7) Hayashi, M et al *In vivo* Rodent Erythrocyte Micronucleus Assay *Mutation Research* 312, 293–304 (1994)

(8) Higashikuni, N and Sutou, S An optimal, generalized sampling time of 30 +/- 6 h after double dosing in the mouse peripheral blood micronucleus test. *Mutagenesis* 10, 313–319 (1995).

(9) Fielder, R J et al Report of British Toxicology Society/UK Environmental Mutagen Society Working Group Dose Setting in *In vivo* Mutagenicity Assays *Mutagenesis* 7, 313–319 (1992)

(10) Hayashi, M et al An Application of Acridine Orange Fluorescent Staining to the Micronucleus Test *Mutation Research* 120, 241–247 (1983)

(11) MacGregor, J T et al A Simple Fluorescent Staining Procedure for Micronuclei and RNA in Erythrocytes Using Hoechst 33258 and Pyronin Y *Mutation Research* 120, 269–275 (1983)

(12) Romagna, F and Staniforth, C D The automated bone marrow micronucleus test *Mutation Research* 213, 91–104 (1989)

(13) Gollapudi, B and McFadden, L G Sample size for the estimation of polychromatic to normochromatic erythrocyte ratio in the bone marrow micronucleus test. *Mutation Research* 347, 97–99 (1995)

(14) Richold, M et al *In vivo* Cytogenetics Assays, In: D J Kirkland (Ed) Basic Mutagenicity Tests, UKEMS Recommended Procedures UKEMS Subcommittee on Guidelines for Mutagenicity Testing Report Part I revised Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp 115–141 (1990)

(15) Lovell, D P et al Statistical Analysis of *In vivo* Cytogenetic Assays In D.J. Kirkland (Ed) Statistical Evaluation of Mutagenicity Test Data UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, Report, Part III Cambridge University Press, Cambridge, New York, Port Chester, Melbourne, Sydney, pp 184–232 (1989)

(16) Heddle, J A et al The Induction of Micronuclei as a Measure of Genotoxicity *Mutation Research* 123 61–118 (1983).

(17) MacGregor, J T et al Guidelines for the Conduct of Micronucleus Assays in Mammalian Bone Marrow Erythrocytes *Mutation Research* 189 103–112 (1987)

(18) MacGregor, J T et al The *In vivo* Erythrocyte Micronucleus Test Measurement at Steady State Increases Assay Efficiency and Permits Integration with Toxicity Studies *Fundamental and Applied Toxicology* 14 513–522 (1990)

(19) MacGregor, J T et al Micronuclei in Circulating Erythrocytes: A Rapid Screen for Chromosomal Damage During Routine Toxicity Testing in Mice In. Developments in Science and Practice of Toxicology. Ed A.W Hayes, R C Schnell and T.S Miya, Elsevier, Amsterdam, pp 555–558 (1983)

United States
Environmental Protection
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Prevention Pesticides
and Toxic Substances
(7101)

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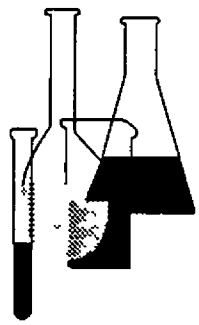
Health Effects Test Guidelines

OPPTS 870.5450
Rodent Dominant Lethal Assay



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S. Environmental Protection Agency under the Toxic Substances Control Act (15 U S C 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.5450 Rodent dominant lethal assay.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5450 Rodent dominant Lethal assay and OECD 478 Genetic Toxicology Rodent Dominant Lethal Assay

(b) **Purpose.** Dominant lethal (DL) effects cause embryonic or fetal death Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definition also applies to this test guideline.

Dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo

(d) **Reference substances.** These may include, but need not be limited to, triethylenemelamine, cyclophosphamide, or ethyl methanesulfonate

(e) **Test method**—(1) **Principle.** Generally, male animals are exposed to the test substance and mated to untreated virgin females The various germ cell stages can be tested separately by the use of sequential mating intervals The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group Pre-implantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups

(2) **Description.** (i) Several treatment schedules are available The most widely used requires single administration of the test substance.

Other treatment schedules, such as treatment on five consecutive days, may be used if justified by the investigator

(ii) Individual males are mated sequentially to virgin females at appropriate intervals The number of matings following treatment is governed by the treatment schedule and should ensure that germ cell maturation is adequately covered Females are sacrificed in the second half of pregnancy and the uterine contents examined to determine the total number of implants and the number of live and dead embryos

(3) **Animal selection**—(i) **Species.** Rats or mice are generally used as the test species Strains with low background dominant lethality, high pregnancy frequency, and high implant numbers are recommended

(ii) **Age.** Healthy, sexually mature animals should be used.

(iii) **Number.** An adequate number of animals should be used taking into account the spontaneous variation of the biological characteristics being evaluated The number chosen should be based on the predetermined sensitivity of detection and power of significance For example, in a typical experiment, the number of males in each group should be sufficient to provide between 30 and 50 pregnant females per mating interval

(iv) **Assignment to groups.** Animals should be randomized and assigned to treatment and control groups

(4) **Control groups**—(i) **Concurrent controls.** Generally concurrent positive and negative (vehicle) controls should be included in each experiment When acceptable positive control results are available from experiments conducted recently (within the last 12 months) in the same laboratory, these results can be used instead of a concurrent positive control

(ii) **Positive controls.** Positive control substances should be used at a dose which demonstrates the test sensitivity

(5) **Test chemicals**—(i) **Vehicle.** When possible, test substances should be dissolved or suspended in isotonic saline or distilled water Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles The vehicle used should neither interfere with the test chemical nor produce toxic effects Fresh preparations of the test chemical should be employed.

(ii) **Dose levels.** Normally, three dose levels should be used The highest dose should produce signs of toxicity (e g., slightly reduced fertility and slightly reduced body weight) However, in an initial assessment of dominant lethality a single high dose may be sufficient Nontoxic substances should be tested at 5 g/kg or, if this is not practicable, then as the highest dose attainable

(iii) **Route of administration.** The usual routes of administration are oral or by IP injection Other routes may be appropriate

(f) **Test performance.** (1) Individual males are mated sequentially at appropriate predetermined intervals to one or two virgin females Females should be left with the males for at least the duration of one estrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug

(2) The number of matings following treatment should be governed by the treatment schedule and should ensure that germ cell maturation is adequately covered

(3) Females should be sacrificed in the second half of pregnancy and uterine contents examined to determine the number of implants and live and dead embryos The ovaries may be examined to determine the number of corpora lutea

(g) **Data and report—(1) Treatment of results.** Data should be tabulated to show the number of males, the number of pregnant females, and the number of nonpregnant females Results of each mating, including the identity of each male and female, should be reported individually For each female, the dose level and week of mating and the frequencies of live implants and of dead implants should be enumerated If the data are recorded as early and late deaths, the tables should make that clear If preplantation loss is estimated, it should be reported Pre-implantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods Differences among animals within the control and treatment groups should be considered before making comparisons between treated and control groups

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of dominant lethals Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of dominant lethals or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) A positive DL assay suggests that under the test conditions the test substance may be genotoxic in the germ cells of the treated sex of the test species

(ii) A negative result suggests that under the conditions of the test the test substance may not be genotoxic in the germ cells of the treated sex of the test species

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Species, strain, age, and weights of animals used, number of animals of each sex in experimental and control groups

(ii) Test substance, vehicle used, dose levels and rationale for dosage selection, negative (vehicle) and positive controls, and experimental observations, including signs of toxicity

(iii) Route and duration of exposure

(iv) Mating schedule

(v) Methods used to determine that mating has occurred (where applicable)

(vi) Criteria for scoring dominant lethals including the number of early and late embryonic deaths

(vii) Dose-response relationship, if applicable

(h) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Brewen, J G et al Studies on chemically induced dominant lethality I The cytogenetic basis of MMS-induced dominant lethality in post-meiotic germ cells. *Mutation Research* 33.239–250 (1975).

(2) Ehling, U H et al Standard protocol for the dominant lethal test on male mice Set up by the Work Group Dominant lethal mutations of the ad hoc Committee Chemogenetics *Archives of Toxicology* 39:173–185 (1978)

United States
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Prevention Pesticides
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(7101)

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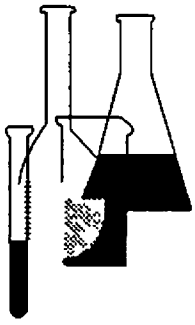
Health Effects Test Guidelines

OPPTS 870.5460
Rodent Heritable Translocation Assays



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U S C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870 5460 Rodent heritable translocation assays.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5460 Rodent heritable translocation assays and OECD guideline 485 Genetic Toxicology Mouse Heritable Translocation Assay

(b) **Purpose.** This test detects transmitted chromosomal damage which manifests as balanced reciprocal translocations in progeny descended from parental males treated with chemical mutagens.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline

Diakinesis and *metaphase* I are stages of meiotic prophase scored cytologically for the presence of multivalent chromosome association characteristic of translocation carriers

Heritable translocation is one in which distal segments of non-homologous chromosomes are involved in a reciprocal exchange.

(d) **Test method**—(1) **Principle.** When a balanced reciprocal translocation is induced in a parental male germ cell, the resulting progeny is translocation heterozygote

(i) **Basis for fertility screening.** Male translocation heterozygotes may be completely sterile This class consists of two types of translocations

(A) Translocations between non-homologous chromosomes in which at least one of the breaks occurs close to one end of a chromosome.

(B) Those that carry multiple translocations The majority of male translocation heterozygotes are semisterile—they carry one or (rarely) two translocations The degree of semisterility is dependent upon the proportions of balanced and unbalanced (duplication-deficiency) gametes produced in the ejaculate as a function of meiotic segregation Balanced and unbalanced sperm are equally capable of fertilizing an egg. Balanced sperm lead to viable progeny Unbalanced sperm result in early embryonic lethality.

(ii) **Basis for cytological screening.** The great majority of male translocation heterozygotes can be identified cytologically through analysis of diakinesis metaphase I spermatocytes Translocation heterozygotes are characterized by the presence of multivalent chromosome association such

as a ring or chain of four chromosomes held together by chiasmata in paired homologous regions Some translocation carriers can be identified by the presence of extra long and/or extra short chromosomes in spermatogonial and somatic cell metaphase preparations

(2) **Description.** Essentially, two methods have been used to screen for translocation heterozygosity—one method uses a mating sequence to identify sterile and semisterile males followed by cytological examination of suspect male individuals, the other method deletes the mating sequence altogether and all F_1 male progeny are examined cytologically for presence of translocation In the former approach, the mating sequence serves as a screen which eliminates most fully fertile animals for cytological confirmation as translocation heterozygotes

(3) **Animal selection**—(i) **Species.** The mouse is the species generally used and is recommended

(ii) **Age.** Healthy sexually mature animals should be used

(iii) **Number.** The number of male animals necessary is determined by the following factors

(A) The use of either historical or concurrent controls

(B) The power of the test

(C) The minimal rate of induction required

(D) Whether positive controls are used

(E) The level of significance desired

(iv) **Assignment to groups.** Animals should be randomized and assigned to treatment and control groups

(4) **Control groups**—(i) **Concurrent controls.** No concurrent positive or negative (vehicle) controls are recommended as routine parts of the heritable translocation assay However, investigators not experienced in performing translocation testing should include a substance known to produce translocations in the assay as a positive control reference chemical.

(ii) **Historical controls.** At the present time, historical control data must be used in tests for significance When statistically reliable historical controls are not available, negative (vehicle) controls should be used

(5) **Test chemicals**—(i) **Vehicle.** When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles The vehicle used should

neither interfere with the test chemical nor produce toxic effects Fresh preparations of the test chemical should be employed

(ii) **Dose levels** At least two dose levels should be used The highest dose level should result in toxic effects (which should not produce an incidence of fatalities which would prevent a meaningful evaluation) or should be the highest dose attainable or 5 g/kg body weight

(iii) **Route of administration.** Acceptable routes of administration include oral, inhalation, admixture with food or water, and IP or IV injection

(e) **Test performance—(1) Treatment and mating.** The animals should be dosed with the test substances 7 days per week over a period of 35 days After treatment, each male should be caged with two untreated females for a period of 1 week At the end of 1 week, females should be separated from males and caged individually. When females give birth, the day of birth, litter size, and sex of progeny should be recorded All male progeny should be weaned, and all female progeny should be discarded

(2) **Testing for translocation heterozygosity.** When males are sexually mature, testing for translocation heterozygosity should begin One of two methods should be used, the first method involves mating, determining those F_1 progeny which are sterile or semisterile and subsequent cytological analysis of suspect progeny, the other method does not involve mating and determining sterility or semisterility, all progeny are examined cytologically.

(i) **Determination of sterility or semisterility—(A) Conventional method.** Females are mated, usually three females for each male, and each female is killed at midpregnancy. Living and dead implantations are counted. Criteria for determining normal and semisterile males are usually established for each new strain because the number of dead implantations varies considerably among strains

(B) **Sequential method.** Males to be tested are caged individually with females and the majority of the presumably normal males are identified on the basis of a predetermined size of one or two litters Breeding pens are examined daily on weekdays beginning 18 days after pairing Young are discarded immediately after they are scored Males that sire a litter whose size is the same as or greater than the minimum set for a translocation-free condition are discarded with their litter. If the litter size is smaller than the predetermined number, a second litter is produced with the same rule applying Males that cannot be classified as normal after production of a second litter are tested further by the conventional method or by cytological confirmation of translocation

(ii) **Cytological analysis.** For cytological analysis of suspected semisteriles, the air-drying technique is used Observation of at least two diakinesis-metaphase I cells with multivalent association constitutes the required evidence for the presence of a translocation Sterile males are examined by one of two methods, those with testes of normal size and sperm in the epididymis are examined by the same techniques used for semisteriles Animals with small testes are examined by squash preparations or, alternatively, by examination of mitotic metaphase preparations If squash preparations do not yield diakinesis-metaphase I cells, analysis of spermatogonia or bone marrow for the presence of unusually long or short chromosomes should be performed

(f) **Data and report—(1) Treatment of results.** (i) Data should be presented in tabular form and should include the number of animals at risk, the germ cell stage treated, the number of partial sterlles and semisteriles (if the fertility test is used), the number of cytogenetically confirmed translocation heterozygotes (if the fertility test is used, report the number of confirmed sterlles and confirmed partial sterlles), the translocation rate, and either the standard error of the rate or the upper 95 percent confidence limit on the rate

(ii) These data should be presented for both treated and control groups Historical or concurrent controls should be specified, as well as the randomization procedure used for concurrent controls

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results in the heritable translocation assay indicate that under the test conditions the test substance causes heritable chromosomal damage in the test species

(ii) Negative results indicate that under the test conditions the test substance does not cause heritable chromosomal damage in the test species

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792 subpart J, the following specific information should be reported

(i) Species, strain, age, weight, and number of animals of each sex in each group

(ii) Test chemical vehicle, route and schedule of administration, and toxicity data

(iii) Dosing regimen, doses tested, and rationale for dosage selection.

(iv) Mating schedule and number of females mated to each male

(v) The use of historical or concurrent controls

(vi) Screening procedure including the decision criteria used and the method by which they were determined

(vii) Dose-response relationship, if applicable

(g) **References.** The following references should be consulted for additional background material on this test guideline

(1) Generoso, W M et al Heritable translocation test in mice *Mutation Research* 76 191–215 (1980)

(2) [Reserved]

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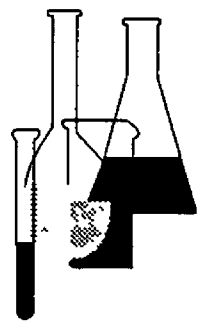
Health Effects Test Guidelines

OPPTS 870.5500
Bacterial DNA Damage or Repair Tests



INTRODUCTION

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OPPTS 870.5500 Bacterial DNA damage or repair tests.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5500 Differential growth inhibition of repair proficient and repair deficient bacteria· Bacterial DNA damage or repair tests and OPP guideline 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982

(b) **Purpose** Bacterial DNA damage or repair tests measure DNA damage which is expressed as differential cell killing or growth inhibition of repair deficient bacteria in a set of repair proficient and deficient strains. These tests do not measure mutagenic events *per se*. They are used as an indication of the interaction of a chemical with genetic material implying the potential for genotoxicity Tests for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA

(c) **Reference substances** These may include, but need not be limited to, chloramphenicol or methyl methanesulfonate

(d) **Test method**—(1) **Principle.** The tests detect agents that interact with cellular DNA to produce growth inhibition or killing. This interaction is recognized by specific cellular repair systems. The assays are based upon the use of paired bacterial strains that differ by the presence of absence of specific DNA repair genes The response is expressed in the preferential inhibition of growth or the preferential killing of the DNA repair deficient strain since it is incapable of removing certain chemical lesions from its DNA

(2) **Description** Several methods for performing the test have been described Those described here are

(i) Tests performed on solid medium (diffusion tests)

(ii) Tests performed in liquid culture (suspension tests)

(3) **Strain selection**—(i) **Designation.** At the present time, *Escherichia coli polA* (W3110/p3478) or *Bacillus subtilis rec* (H17/M45) pairs are recommended. Other pairs may be utilized when appropriate

(ii) **Preparation and storage** Stock culture preparation and storage, growth requirements, method of strain identification, and demonstration

of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented

(4) **Bacterial growth** Good microbiological techniques should be used to grow fresh cultures of bacteria The phase of growth and cell density should be documented and should be adequate for the experimental design

(5) **Metabolic activation** Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system The most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents The use of other species, tissues, or techniques may also be appropriate

(6) **Control groups**—(i) **Concurrent controls.** Concurrent positive, negative, and vehicle controls should be included in each assay

(ii) **Negative controls** The negative control should show nonpreferential growth inhibition (i e , should affect both strains equally) Chloramphenicol is an example of a negative control

(iii) **Genotype specific controls** Examples of genotype specific positive controls are methyl methanesulfonate for *polA* strains and mitomycin C for *rec* strains

(iv) **Positive controls to ensure the efficacy of the activation system.** The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test

(v) **Other positive controls** Other positive control reference substances may be used

(e) **Test chemicals**—(1) **Vehicle** Test chemicals and positive and negative control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay

(2) **Exposure concentrations** The test should initially be performed over a broad range of concentrations Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis Because results are expressed as diameters of zones of growth inhibition in the diffusion test, it is most important that the amounts of chemical on the disc (or in the wells) are exact replicates When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations

(f) **Test performance**—(1) **Diffusion assay**—(i) **Disc diffusion assays.** Disc diffusion assays may be performed in two ways

(A) A single strain of bacteria may be added to an agar overlay or spread on the surface of the agar and the test chemical placed on a filter disc on the surface of the agar

(B) DNA repair proficient and DNA repair deficient bacteria may be streaked in a line on the surface of the agar of the same plate and a disc saturated with test chemical placed on the surface of the agar in contact with the streaks

(ii) **Well diffusion assays.** In well diffusion assays, bacteria may be either added to the agar overlay or spread onto the surface of the agar A solution of the test chemical is then placed into a well in the agar

(2) **Suspension assays.** (i) A bacterial suspension may be exposed to the test chemical and the number of surviving bacteria determined (as colony-forming units) either as a function of time of treatment or as a function of the concentration of test agent

(ii) Nonturbid suspensions of bacteria may be exposed to serial dilutions of the test agent and a minimal inhibitory concentration for each strain determined, as evidenced by the presence or absence of visible growth after a period of incubation

(iii) Paired bacterial suspensions (usually with some initial turbidity) may be treated with a single dose of the chemical Positive results are indicated by a differential inhibition in the rate of increase of turbidity of the paired cultures.

(3) **Number of cultures.** When using a plate diffusion procedure, at least two independent plates should be used at each dilution In liquid suspension assays, at least two independent specimens for determination of the number of viable cells should be plated

(4) **Incubation conditions.** All plates in a given test should be incubated for the same time period This incubation period should be for 18 to 24 hours at 37 °C

(g) **Data and report**—(1) **Treatment of results**—(i) **Diffusion assays.** Results should be expressed in diameters of zones of growth inhibition in millimeters or as areas derived therefrom as square millimeters. Dose-response data, if available, should be presented using the same units

(ii) **Liquid suspension assays.** (A) Survival data can be presented as dose responses, preferably as percentage of survivors or fractional survival of each strain or as a relative survival (ratio) of the two strains

(B) Results can also be expressed as the concentrations required to effect a predetermined survival rate (e g , D_{37}, the dose permitting 37 percent survival) These data are derived from the survival curve The concentration should be expressed as weight per volume, as moles, or as molarity

(C) Similarly, results can be expressed as minimal inhibitory concentration or as minimal lethal dose The former is determined by the absence of visible growth in liquid medium and the latter is determined by plating dilutions onto semisolid media

(iii) In all tests, concentrations must be given as the final concentrations during the treatment Raw data, prior to transformation, should be provided These should include actual quantities measured, e.g , neat numbers. For measurement of diffusion, the diameters of the discs and/or well should be indicated and the measurements should indicate whether the diameter of the discs and/or well was subtracted Moreover, mention should be made as to whether the test chemical gave a sharp, diffuse, or double-zone of growth inhibition. If it is the latter, the investigator should indicate whether the inner or the outer zone was measured

(iv) Viability data should be given as the actual plate counts with an indication of the dilution used and the volume plated or as derived titers (cells per milliliter) Transformed data alone in the absence of experimental data are not acceptable (i e, ratios, differences, survival fraction)

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related preferential inhibition or killing of the repair deficient strain. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related preferential inhibition or killing of the repair deficient strain or a statistically significant and reproducible positive response at any one of the test points is considered not to interact with the genetic material of the organisms used in assay

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** DNA damage tests in bacteria do not measure DNA repair per se nor do they measure mutations. They measure DNA damage which is expressed as cell killing or growth inhibition. A positive result in a DNA damage test in the absence of a positive result in another system is difficult to evaluate in the absence of a better data base

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J the following specific information should be reported

(i) Bacterial strains used

(ii) Phase of bacterial cell growth at time of use in the assay

(iii) Media composition

(iv) Details of both the protocol used to prepare the metabolic activation system and its use in the assay

(v) Treatment protocol, including doses used and rationale for dose selection, positive and negative controls

(vi) Method used for determination of degree of cell kill

(vii) Dose-response relationship, if applicable

(g) **References.** The following references should be consulted for additional background material on this test guideline.

(1) Ames, B N et al Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test *Mutation Research* 31.347–364 (1975)

(2) Kada, T et al In vitro and host-mediated rec-assay procedures for screening chemical mutagens, and phloxine, a mutagenic red dye detected *Mutation Research* 16 165–174 (1972)

(3) Leifer, Z et al An evaluation of bacterial DNA repair tests for predicting genotoxicity and carcinogenicity A report of the U S. EPA's Gene-Tox Program *Mutation Research* 87 211–297 (1981)

(4) Slater, E E et al Rapid detection of mutagens and carcinogens. *Cancer Research* 31 970–973 (1971)

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(7101)

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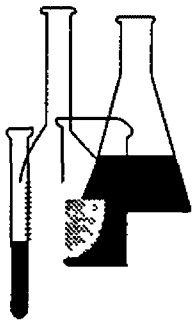
Health Effects Test Guidelines

OPPTS 870.5550 Unscheduled DNA Synthesis in Mammalian Cells in Culture



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OPPTS 870.5550 Unscheduled DNA synthesis in mammalian cells in culture.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5550 Unscheduled DNA synthesis in mammalian cells in culture and OECD guideline 482 Genetic Toxicology DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells in Vitro

(b) **Purpose.** Unscheduled DNA synthesis (UDS) in mammalian cells in culture measures the repair of DNA damage induced by a variety of agents including chemicals, radiation and viruses UDS may be measured in both *in vitro* and *in vivo* systems

(c) **Definition.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definition also applies to this test guideline

Unscheduled DNA synthesis in mammalian cells in culture is the incorporation of tritium-labeled thymidine (^{3}H-TdR) into the DNA of cells which are not in the S phase of the cell cycle

(d) **Reference substances.** These may include, but need not be limited to, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 4-nitroquinoline oxide or *N*-dimethylnitrosamine

(e) **Test method**—(1) **Principle.** Mammalian cells in culture, either primary cultures of rodent hepatocytes or established cell lines, are exposed to the test agent. Established cell lines are treated both with and without metabolic activation UDS is measured by the uptake of ^{3}H-TdR into the DNA of non-S phase cells. Uptake may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from treated cells

(2) **Description**—(i) **Autoradiography.** For autoradiography, coverslip cultures of cells are exposed to test chemical in medium containing ^{3}H-TdR. At the end of the treatment period, cells are fixed, dipped in autoradiographic emulsion, and exposed at 4 °C At the end of the exposure period, cells are stained and labeled nuclei are counted either manually or with an electronic counter Established cell lines should be treated both with and without metabolic activation.

(ii) **LSC determinations** For LSC determinations of UDS, confluent cultures of cells are treated with test chemical both with and without metabolic activation At the end of the exposure period, DNA is extracted from

the treated cells Total DNA content is determined and extent of ^{3}H-TdR incorporation is determined by scintillation counting

(3) **Cells—(i) Type of cells used in the assay.** (A) A variety of cell lines or primary cell cultures, including human cells, may be used in the assay

(B) Established cell lines should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability

(ii) **Cell growth and maintenance** Appropriate culture media and incubation conditions (culture vessels CO_2 concentration), temperature, and humidity should be used

(4) **Metabolic activation** (i) A metabolic activation system is not used with primary cultures of rodent hepatocytes

(ii) Established cell lines should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system

(5) **Control groups** Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation as appropriate should be included in each experiment

(6) **Test chemicals—(i) Vehicle.** Test chemicals and positive control reference substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells Final concentration of the vehicle should not interfere with cell viability or growth rate

(ii) **Exposure concentrations** Multiple concentrations of test substance, based upon cytotoxicity and over a range adequate to define the response, should be used For cytotoxic chemicals, the first dose to elicit a cytotoxic response in a preliminary assay should be the highest dose tested Relatively insoluble compounds should be tested up to the limits of solubility For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis

(f) **Test performance—(1) Primary cultures of rodent hepatocytes.** Freshly isolated rodent hepatocytes should be treated with chemical in medium containing ^{3}H-TdR At the end of the treatment period, cells should be drained of medium, rinsed, fixed, dried, and attached to microscope slides Slides should be dipped in autoradiographic emulsion, exposed at 4 °C for an appropriate length of time, developed, stained, and counted

(2) **Established cell lines—(i) Autoradiographic techniques.** The techniques for treatment of established cell lines are the same as those for primary cultures of rodent hepatocytes except that cells must not enter S phase prior to treatment Entry of cells into S phase may be blocked by several methods (e g , by growth in medium deficient in arginine or

low in serum or by treatment with chemical agents such as hydroxyurea) Tests should be done both in the presence and absence of a metabolic activation system

(ii) **LSC measurement of UDS** Prior to treatment with test agent, entry of cells into S phase should be blocked as described in paragraph (f)(2)(i) of this guideline Cells should be exposed to the test chemical in medium containing ^{3}H-TdR At the end of the incubation period, DNA should be extracted from the cells by hydrolysis with perchloroacetic acid or by other acceptable methods One aliquot of DNA is used to determine total DNA content, a second aliquot is used to measure the extent of ^{3}H-TdR incorporation

(3) **Acceptable background frequencies—(i) Autoradiographic determinations** Net incorporation of ^{3}H-TdR into the nucleus of solvent treated control cultures should be less than 1

(ii) **LSC determinations** Historical background incorporation rates of ^{3}H-TdR into untreated established cell lines should be established for each laboratory

(4) **Number of cells counted** A minimum of 50 cells per culture should be counted for autoradiographic UDS determinations. Slides should be coded before being counted Several widely separated random fields should be counted on each slide Cytoplasm adjacent to the nuclear areas should be counted to determine spontaneous background.

(5) **Number of cultures** Six independent cultures at each concentration and control should be used in LSC UDS determinations

(g) **Data and report—(1) Treatment of results—(i) Autoradiographic determinations.** For autoradiographic determinations, once untransformed data are recorded, background counts should be subtracted to give the correct nuclear grain count Values should be reported as net grains per nucleus Mean, median, and mode may be used to describe the distribution of net grains per nucleus

(ii) **LSC determinations** For LSC determinations, ^{3}H-TdR incorporation should be reported as disintegrations per minute per microgram of DNA. Average disintegrations per minute per microgram of DNA with standard deviation or standard error of the mean may be used to describe distribution of incorporation in these studies

(2) **Statistical evaluation** Data should be evaluated by appropriate statistical methods.

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the incorporation of ^{3}H-TdR into treated cells. Another

criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the incorporation of ^{3}H-TdR into treated cells or a statistically significant and reproducible positive response at any one of the test points is considered not to induce UDS in the test system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results in the UDS assay indicate that under the test conditions the test substance may induce DNA damage in cultured mammalian somatic cells

(ii) Negative results indicate that under the test conditions the test substance does not induce DNA damage in cultured mammalian somatic cells.

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Cells used, density and passage number at time of treatment, number of cell cultures

(ii) Methods used for maintenance of cell cultures including medium, temperature, and CO_2 concentration

(iii) Test chemical vehicle, concentration, and rationale for selection of concentrations used in the assay

(iv) Details of both the protocol used preparation of the metabolic activation system and its use in the assay.

(v) Treatment protocol

(vi) Positive and negative controls

(vii) Protocol used for autoradiography

(viii) Details of the method used to block entry of cells into S phase.

(ix) Details of the methods used for DNA extraction and determination of total DNA content in LSC determinations

(x) Historical background incorporation rates of ^{3}H-TdR in untreated cell lines

(xi) Dose-response relationship, if applicable

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Ames, B N et al Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test *Mutation Research* 31 347–364 (1975)

(2) Rasmussen, R E and Painter, R B Radiation-stimulated DNA synthesis in cultured mammalian cells *Journal of Cell Biology* 29 11–19 (1966)

(3) Stich, H F et al DNA fragmentation and DNA repair as an in vitro and in vivo assay for chemical procarcinogens, carcinogens and carcinogenic nitrosation products, Screening tests in chemical carcinogenesis Eds Bartsch, H , Tomatis, L IARC Scientific, Lyon, No 12 (1976) pp 617–636

(4) Williams, G M Carcinogen-induced DNA repair in primary rat liver cell cultures a possible screen for chemical carcinogens *Cancer Letters* 1 231–236 (1976)

(5) Williams, G M Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures *Cancer Research* 37 1845–1851 (1977)

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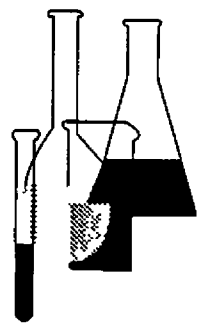
Health Effects Test Guidelines

OPPTS 870.5575
Mitotic Gene Conversion in *Saccharomyces cerevisiae*



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OPPTS 870.5575 Mitotic gene conversion in Saccharomyces cerevisiae.

(a) **Scope**—(1) **Applicability** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798.5575 Mitotic gene gene conversion in Saccharomyces cerevisiae and OPP 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982

(b) **Purpose.** The mitotic gene conversion assay in the yeast, *Saccharomyces cerevisiae* (*S cerevisiae*), measures the conversion of differentially inactive alleles to wild-type alleles by mutagenic agents Heteroallelic diploid yeast strains carry two different inactive alleles of the same gene locus The presence of these alleles causes a nutritional requirement, e g , these heteroallelic diploids grow only in medium supplemented with a specific nutrient such as tryptophan When gene conversion occurs, a fully active wild-type phenotype is produced from these inactive alleles through intragenic recombination These wild-type colonies grow on a medium lacking the specific nutritional requirement (selective medium)

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definitions also apply to this test guideline.

Heteroallelic diploids are diploid strains of yeast carrying two different, inactive alleles of the same gene locus causing a nutritional requirement

Mitotic gene conversion is detected by the change of inactive alleles of the same gene to wild-type alleles through intragenic recombination in mitotic cells

(d) **Reference substances.** These may include, but need not be limited to, hydrazine sulfate or 2-acetylaminofluorene

(e) **Test method**—(1) **Principle.** The method is based on the fact that heteroallelic diploid yeast strains carry two inactive alleles of the same gene locus making them dependent on a specific nutritional requirement (e g., tryptophan) for their survival Treatment of such strains with mutagenic agents can cause conversion of these alleles back to the wild-type condition which allows growth on a medium lacking the required nutrient (selective medium)

(2) **Description.** Heteroallelic diploid strains such as D7, requiring a specific nutrient in the medium are treated with test chemical with and without metabolic activation and plated on a selective medium lacking the required nutrient The wild-type colonies that grow on the selective medium as a result of gene conversion are scored

(3) **Strain selection—(i) Designation.** At the present time, *S cerevisiae* strain D7 is recommended for use in this assay The use of other strains may also be appropriate

(ii) **Preparation and storage.** Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented

(iii) **Media.** YEP glucose medium enriched with the appropriate growth factors may be used for cell growth and maintenance Other media may also be appropriate

(4) **Selection of cultures.** Cells should be grown with aeration in liquid medium enriched with growth factors to early stationary phase Cells should then be seeded on selective medium to determine the rate of spontaneous conversion Cultures with a high rate of spontaneous conversion should be discarded

(5) **Metabolic activation.** Cells should be exposed to test chemical both in the presence and absence of an appropriate metabolic activation system.

(6) **Control groups.** Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment

(7) **Test chemicals—(i) Vehicle.** Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay. Dimethylsulfoxide should be avoided as a vehicle

(ii) **Exposure concentrations.** (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems For cytotoxic chemicals, the highest dose tested should not reduce survival to less than 10 percent of that seen in the untreated control cultures. Relatively insoluble chemicals should be tested up to the limits of solubility For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case-by-case basis

(B) When appropriate, a positive response should be confirmed by using a narrow range of concentrations

(f) **Test performance**—(1) **Treatment.** Cultures should be treated in liquid suspension Resting cells should be treated in buffer, growing cells should be treated in a synthetic medium Cultures with low spontaneous convertant frequencies should be centrifuged, washed and resuspended in liquid at the appropriate density Cells should be exposed to test chemical both in the presence and absence of a metabolic activation system Independent tubes should be treated for each concentration At the end of the treatment period, cells should be centrifuged, washed and resuspended in distilled water prior to plating on selective medium for convertant selection and on complete medium to determine survival At the end of the incubation period, plates should be scored for survival and the presence of convertant colonies

(2) **Number of cultures.** At least six individual plates per treatment concentration and control should be used

(3) **Incubation conditions.** All plates in a given experiment should be incubated for the same time period This incubation period may be from 4 to 6 days at 28 °C

(g) **Data and report**—(1) **Treatment of results.** Individual plate counts for test substance and control should be presented for both convertants and survivors The mean number of colonies per plate and standard deviation should also be presented Data should be presented in tabular form indicating numbers of viable and convertant colonies scored, survival frequency and convertant frequencies for each treatment and control culture Conversion frequencies should be expressed as number of convertants per number of survivors Sufficient detail should be provided for verification of survival and convertant frequencies

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of gene convertants Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of gene conversions or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results in this assay indicate that under the test conditions the test chemical causes mitotic gene conversion in the yeast *S cerevisiae*

(ii) Negative results indicate that under the test conditions the test chemical does not cause mitotic gene conversion in *S cerevisiae*

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Strain of organism used in the assay

(ii) Test chemical vehicle, doses used, and rationale for dosage selection

(iii) Method used to select cultures

(iv) Treatment protocol including cell density at treatment and length of exposure to test substance

(v) Details of both the protocol used to prepare the metabolic activation system and its use in the assay

(vi) Incubation times and temperatures

(vii) Dose-response relationship, if applicable

(h) **References.** The following references should be consulted for additional background material on this test guideline

(1) Ames, B.N et al Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test *Mutation Research* 31 347–364 (1975)

(2) Callen, D F and Philpot, R M Cytochrome P–450 and the activation of promutagens in *Saccharomyces cerevisiae Mutation Research* 45.309–324 (1975)

(3) Zimmermann, F K Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. Handbook of mutagenicity test procedures Eds Kilby, B.J , Legator, M , Nicols, W , Ramel, C Elsevier/North Holland Biomedical Press, Amsterdam (1979) pp. 119–134.

(4) Zimmermann, F.K et al A yeast strain for simultaneous detection of induced mitotic crossing over, mitotic gene conversion and reverse mutation *Mutation Research* 28·381–388 (1975)

United States
Environmental Protection
Agency

Prevention Pesticides
and Toxic Substances
(7101)

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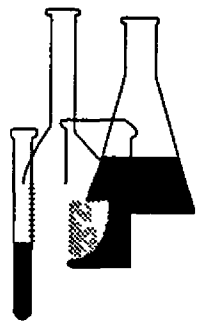
Health Effects Test Guidelines

OPPTS 870.5900
In Vitro Sister Chromatid Exchange Assay



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD)

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U S Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U S C 136, *et seq*)

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OPPTS 870.5900 In vitro sister chromatid exchange assay

(a) **Scope**—(1) **Applicability** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798 5900 In vitro sister chromatid exchange assay and OECD 479 Genetic Toxicology. In Vitro Sister Chromatid Exchange Assay in Mammalian Cells

(b) **Purpose.** The sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome The test may be performed *in vitro*, using, for example, rodent or human cells, or *in vivo* using mammals, for example, rodents such as mice, rats and hamsters

(c) **Definitions** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definition also applies to this test guideline

Sister chromatid exchanges are reciprocal interchanges of the two chromatid arms within a single chromosome These exchanges are visualized during the metaphase portion of the cell cycle and presumably require enzymatic incision, translocation and ligation of at least two DNA helices.

(d) **Test method**—(1) **Principle.** Following exposure of cell cultures to test chemicals, they are allowed to replicate in the presence of bromodeoxyuridine (BrdU), followed by treatment with colchicine or colcemid to arrest cells in a metaphase-like stage of mitosis (c-metaphase). Cells are then harvested and chromosome preparations made Preparations are stained and metaphase cells analyzed for SCEs

(2) **Description.** *In vitro* SCE assays may employ monolayer or suspension cultures of established cell lines, cell strains, or primary cell cultures. Cell cultures are exposed to test chemical and are allowed to replicate in the presence of BrdU Prior to harvest, cells are treated with a spindle inhibitor (e g , Colchicine or Colcemid®) to accumulate cells in c-metaphase. Chromosome preparations from cells are made, stained, and analyzed for SCEs

(3) **Cells**—(i) **Type of cells used in the assay.** There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay Established cell lines and strains should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability

(ii) **Cell growth and maintenance.** Appropriate culture media and incubation conditions (culture vessels, temperature, humidity, and CO_2 concentration) should be used

(4) **Metabolic activation.** Cells should be exposed to test chemical both in the presence and absence of an appropriate metabolic activation system

(5) **Control groups—Concurrent controls.** Positive and negative (untreated and/or vehicle) controls, with and without metabolic activation, should be included in each experiment

(6) **Test chemicals—(i) Vehicle.** Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells Final concentration of the vehicle should not reduce cell viability or growth rate

(ii) **Exposure concentrations.** Multiple concentrations of the test substance over a range adequate to define the response should be tested Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test substance may be altered in the presence of metabolic activation systems Cytotoxicity may be evidenced by a large (e g , 75 percent) decrease in the number of cells that have divided twice in the presence of BrdU Relatively insoluble substances should be tested up to the limit of solubility For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. When appropriate, a positive response should be confirmed by using a narrow range of test concentrations

(e) **Test performance—(1) Established cell lines and strains.** (i) Prior to use in the assay, cells should be generated from stock cultures, seeded in culture vessels at the appropriate density and incubated at 37 °C

(ii) Cell lines and strains should be treated with test chemical both with and without metabolic activation when they are in the exponential stage of growth At the end of the exposure period, cells should be washed and incubated for two replication cycles in medium containing BrdU After BrdU is added, the cultures should be handled in darkness, under "safe" (e g , darkroom) lights, or in dim light from incandescent lamps to minimize photolysis of BrdU containing DNA At the end of the BrdU incubation period, cells should be fixed and stained for SCE determination Cultures should be treated with a spindle inhibitor (e.g., colchicine or Colcemid ®) 2 hours prior to harvesting

(2) **Human lymphocyte cultures.** (i) For preparation of human lymphocyte cell cultures, heparinized or acid-citrate-dextrose treated whole blood should be added to culture medium containing a mitogen, e.g ,

phytohemagglutinin (PHA) and incubated at 37 °C White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient such as Ficoll-Hypaque may also be utilized

(ii) Cells should be exposed to the test chemical during at last two time intervals, e g , G_0 and S Exposure during the G_0 phase of the cell cycle should be accomplished by adding the test substance prior to addition of mitogen Exposure during or after the first S phase may be accomplished by exposing cells 24–30 hours after mitosis, under "safe" (e g , darkroom) lights, or in dim light from incandescent lamps to minimize photolysis of BrdU containing DNA At the end of the BrdU incubation period, cells should be fixed and stained for SCE determination Cultures should be treated with a spindle inhibitor (e g., colchicine or Colcemid®) 2 hours prior to harvesting

(3) **Human lymphocyte cultures.** (i) For preparation of human lymphocyte cell cultures, heparinized or acid-citrate-dextrose treated whole blood should be added to culture medium containing a mitogen, e g , phytohemagglutinin (PHA) and incubated at 37 °C White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient such as Ficoll-Hypaque may also be utilized

(ii) Cells should be exposed to the test chemical during at least two time intervals, e g , G_0 and S Exposure during the G_0 phase of the cell cycle should be accomplished by adding the test substance prior to addition of mitogen. Exposure during or after the first S phase may be accomplished by exposing cells 24–30 hours after mitogen stimulation After exposure, cells should be washed and then cultured in the absence of the chemical

(4) **Culture harvest time.** A single harvest time, one that yields an optimal percentage of second division metaphases, is recommended If there is reason to suspect that this is not a representative sampling time (which may occur for short-lived, cycle specific chemicals), then additional harvest times should be selected

(5) **Staining method.** Staining of slides to reveal SCEs can be performed according to any of several protocols However, the fluorescence plus Giemsa method is recommended

(6) **Number of cultures.** At least two independent cultures should be used for each experimental point

(7) **Analysis.** Slides should be coded before analysis The number of cells to be analyzed should be based upon the spontaneous control frequency and defined sensitivity and the power of the test chosen before analysis In human lymphocytes, only cells containing 46 centromeres should be analyzed In established cell lines and strains, only metaphases

containing ±2 centromeres of the modal number should be analyzed Uniform criteria for scoring SCEs should be used

(f) **Data and report**—(1) **Treatment of results** Data should be presented in tabular form, providing scores for both the number of SCEs for each metaphase and the number of SCEs per chromosome for each metaphase

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods.

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of sister chromatid exchanges Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations

(ii) A test substance which produces neither a statistically significant dose-related increase in the number of sister chromatid exchanges nor a statistically significant and reproducible positive response at any one of the test points is considered not to induce rearrangements of segments of DNA in this system

(iii) Both biological and statistical significance should be considered together in the evaluation

(4) **Test evaluation.** (i) Positive results in the *in vitro* SCE assay indicate that under the test conditions the test substance induces reciprocal chromatid interchanges in cultured mammalian somatic cells

(ii) Negative results indicate that under the test conditions the test substance does not induce reciprocal chromatid interchanges in cultured mammalian somatic cells

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported.

(i) Cells used, density at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature, and CO_2 concentration

(iii) Test chemical vehicle, concentration and rationale for the selection of the concentrations of test chemical used in the assay, duration of treatment

(iv) Details of both the protocol used preparation of the metabolic activation system and its use in the assay

(v) Growth period in BrdU, identity of spindle inhibitor, its concentration and duration of treatment

(vi) Time of cell harvest

(vii) Positive and negative controls

(viii) Method used to prepare slides for SCE determination

(ix) Criteria for scoring SCEs

(x) Details of the protocol used for growth and treatment of human cells if used in the assay

(xi) Dose-response relationship, if applicable

(g) **References.** The following references should be consulted for additional background material on this test guideline

(1) Latt, S A et al Sister chromatid exchanges a report of the U.S EPA's Gene-Tox Program *Mutation Research* 87 17–62 (1981)

(2) [Reserved]

United States
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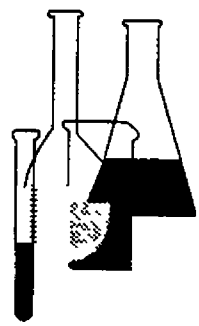
Health Effects Test Guidelines

OPPTS 870.5915
In Vivo Sister Chromatid Exchange Assay



INTRODUCTION

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OPPTS 870.5915 In vivo sister chromatid exchange assay.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U S C 136, *et seq*) and the Toxic Substances Control Act (TSCA) (15 U S C 2601)

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are OPPT 40 CFR 798.5915 In vivo sister chromatid exchange assay and OPP 84–2 Mutagenicity Testing (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation, Human and Domestic Animals) EPA report 540/09–82–025, 1982

(b) **Purpose.** The sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome The test may be performed *in vitro* using cultured mammalian cells or *in vivo* using nonmammalian or mammalian tissues The most commonly used assays employ bone marrow or lymphocytes from mammalian species such as mice, rats, or hamsters Human lymphocytes may also be used

(c) **Definition.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline The following definition also applies to this test guideline

Sister chromatid exchanges are reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges are visualized during the metaphase portion of the cell cycle and presumably require enzymatic incision, translocation and ligation of at least two DNA helices.

(d) **Test method**—(1) **Principle.** Animals are exposed to test substance by appropriate routes followed by administration of bromodeoxyuridine (BrdU) A spindle inhibitor (e g , colchicine or Colcemid®) is administered prior to sacrifice After sacrifice, tissue is obtained and metaphase preparations made, stained, and scored for SCE

(2) **Description.** The method described here employs bone marrow of laboratory rodents exposed to test chemicals. After treatment with test chemical, animals are further treated with BrdU and, prior to sacrifice, with a spindle inhibitor (e g , colchicine or Colcemid®) to arrest cells in c-metaphase After sacrifice, chromosome preparations from bone marrow cells are made, stained, and scored for SCE

(3) **Animal selection**—(i) **Species and strain.** Any appropriate mammalian species may be used Examples of commonly used rodent species include mice, rats, and hamsters

(ii) **Age.** Healthy, young adult animals should be used

(iii) **Number and sex.** At least five female and five male animals per experimental and control group should be used The use of a single sex or different number of animals should be justified

(iv) **Assignment to groups.** Animals should be randomized and assigned to treatment and control groups

(4) **Control groups**—(i) **Concurrent controls.** Current positive and negative (vehicle) controls should be included in the assay

(ii) **Positive controls.** A compound know to produce SCE *in vivo* should be employed as the positive control

(5) **Test chemicals**—(i) **Vehicle.** When possible, test chemicals should be dissolved in isotonic saline or distilled water Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test compound nor produce toxic effects Fresh preparations of the test compound should be employed

(ii) **Dose levels.** For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose or that producing some indication of toxicity as evidenced by animal morbidity (including death) or target cell toxicity The LD_{50} is a suitable guide Additional dose levels may be used For determination of dose-response, at least three dose levels should be used

(iii) **Route of administration.** The usual routes of administration are IP or oral Other routes may be appropriate

(iv) **Treatment schedule.** In general, test substances should be administered only once However, based upon toxicological information a repeated treatment schedule may be employed

(e) **Test performance**—(1) **Treatment.** Animals should be treated with test chemical followed by administration of BrdU. BrdU may be administered by multiple IP injections, by continuous tail vein infusion or by subcutaneous implantation of tablets Animals should be treated with a spindle inhibitor (e g , colchicine or Colcemid®) 2 hours prior to sacrifice After sacrifice, bone marrow should be extracted and slides made and prepared for SCE evaluation

(2) **Staining method.** Staining of slides to reveal SCEs can be performed according to any of several protocols However, the fluorescence plus Giemsa method is recommended

(3) **Number of cells scored.** The number of cells to be analyzed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity and the power chosen for the test Slides should be coded before microscopic analysis

(f) **Data and report**—(1) **Treatment of results.** Data should be presented in tabular form, providing scores for both the number of SCE for each metaphase and the number of SCE per chromosome for each metaphase Differences among animals within each group should be considered before making comparisons between treated and control groups

(2) **Statistical evaluation.** Data should be evaluated by appropriate statistical methods

(3) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of SCE Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of SCE or a statistically significant and reproducible positive response at any one of the test points is considered not to induce rearrangements of DNA segments in this system

(iii) Both biological and statistical significance should be considered in the evaluation

(4) **Test evaluation.** (i) Positive results in the *in vivo* SCE assayindicate that under the test conditions the test substance induces reciprocal interchanges in the bone marrow of the test species

(ii) Negative results indicate that under the test conditions the test substance does not induce reciprocal interchanges in the bone marrow of the test species

(5) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported

(i) Species, strain, age, weight, number, and sex of animals in each treatment and control group.

(ii) Test chemical vehicle, dose level used, rationale for dose selection, toxicity data, negative and positive controls

(iii) Route and schedule of administration of both test chemical and BrdU

(iv) Identity of spindle inhibitor, its concentration and duration of treatment

(v) Time of sacrifice after administration of BrdU

(vi) Details of the protocol used for slide preparation

(vii) Criteria for scoring SCE

(viii) Dose-response relationship if applicable

(g) **References.** The following references should be consulted for additional background material on this test guideline

(1) Allen, J W et al Bromodeoxyuridine tablet methodology for in vivo studies of DNA synthesis *Somatic Cell Genetics* 4 393–405 (1978)

(2) Allen, J W et al Simplified technique for in vivo analysis of sister chromatid exchanges using 5-bromodeoxyuridine tablets *Cytogenetics Cell Genetics* 18 231–237 (1977)

(3) Latt, S A et al Sister chromatid exchanges A report of the U.S. EPA Gene-Tox Program *Mutation Research* 87 17–62 (1981).